SUSCEPTIBILITY OF MULTIDRUG RESISTANT CLINICAL ISOLATES TO ANTISEPTICS, DISINFECTANTS, HERBAL PRODUCTS AND EFFECT OF ITS EXPOSURE TO THE SAME ON THE DEVELOPMENT OF ANTIBIOTIC RESISTANCE IN THE TEST ISOLATES

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Certificate

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CERTIFICATE

This is to certify that the dissertation work entitled "**Susceptibility of multidrug resistant clinical isolates to antiseptics, disinfectants, herbal products and effect of its exposure to the same on the development of antibiotic resistance in the test isolates" submitted** by **Dr. D.Lavanya** and this work was done by her during the period of study in this department from January 2015 to July 2016. This work was done under direct guidance of **Dr. Marina Thomas,** Professor, Department of Microbiology, PSG IMS & R.

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1.Introduction

Development of multidrug resistant isolates *Pseudomonas aeruginosa* and *Acinetobacter* spp and their easy dissemination in hospital environment have compounded the morbidity of already sick people in the hospital and are also increasing their mortality. These are also the predominant bacteria present in the hospital environment. Both *Pseudomonas* and *Acinetobacter* spp have extraordinary potential to form biofilms which serve as reservoirs of bacteria and they contribute to their resistance to antibiotics and disinfectants[1]. *Pseudomonas* spp possess active efflux pump system which acts as wide transporters for disinfectants and also they possess Porin channels which are narrow thereby restricting the entry of antibiotics inside.

Hospital environment as we know is always exposed to surface disinfectants for the purpose of keeping environmental contaminants away from patients and devices. Patients we know are also pumped with antibiotics to contain presumed bacterial multiplication. In this scenario how does a bacteria respond? Is the response only to the antibiotics exposed or also to the disinfectants and antiseptics present in the hospital environment? Does this multiple exposure have any specific effect on the bacterial population? Does it have any effect on the MIC levels? Is the optimum concentration of disinfectants used in hospital crucial? Isolated reports and speculations are described in literature that there is a massive increase in MIC levels of these antibiotics, especially when disinfectant concentrations below optimum are used![4].Today *Acinetobacter* spp have been defined as red alert pathogens due to their resistance to extensive antibiotics. *Pseudomonas* is already identified as inherently resistant to some of the

disinfectants. These two together are important hospital acquired pathogens gaining importance by the day. How do we deal with these pathogens?

Neem extract has antibacterial, antifungal and antiviral effects and is used to treat ulcers and superficial skin infections[2]. Each and every part of Neem tree is said to possess a unique property. Twigs from Neem tree are widely used as chewing sticks in the Indian subcontinent[3] . Is the antimicrobial activity of Neem leaves against *Pseudomonas aeruginosa* and *Acinetobacter baumannii* also affected by exposure of these isolates to disinfectants in below optimum concentrations? This study answers some of the questions posed.

2. Aims and Objectives

AIM

To study the effect of exposure to antiseptics, disinfectants and herb(Neem leaves) on antibiotic susceptibility of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

OBJECTIVES

1) To determine the MIC of drugs such as Gentamicin, Ciprofloxacin, and Imipenem to the selected clinical isolates by Agar dilution method.
2) To expose the clinical isolates to varying concentrations of Sodium Hypochlorite and Benzalkonium chloride.
3) To compare the MIC of above mentioned antibiotics to the clinical isolates before and after exposure to antiseptics and disinfectants.
4) To prepare Neem leaf extract and to determine the susceptibility of the selected isolates to the prepared extract before and after exposure to antiseptics and disinfectants.

3. Review of Literature

ANTIBIOTICS

Antibiotics, also called antimicrobials are probably one of the most successful forms of chemotherapy in the history of medicine [1]. They act against bacteria and prevent bacterial infections through destruction of organisms and also by altering the environment necessary for bacterial growth and multiplication. They do not act against viruses. Antimicrobials can be classified based on the spectrum of action, route of administration and mechanism of action. Based on the spectrum, they are divided into Broad spectrum antibiotics and Narrow spectrum antibiotics. The former act against a broader range of bacteria in comparison to the latter. Based on the mechanism, they can be classified into Bactericidal and Bacteriostatic. The former kills the bacteria whereas the latter halt the growth of the microorganisms.

HISTORY OF ANTIBIOTICS

In the pre-antibiotic era, the methods of treating infections were entirely different. For example, moulds were used to treat infected wounds in India whereas Greeks used lukewarm soil for treating wound infection. In Babylonia, ocular infections were treated using a mixture of milk and bile fluid obtained from frogs. Antimicrobial exposure in the ancient period could be through traditional or alternative medicine. The well known example was the most effective anti-malarial drug, qinghaosu (artemisinin) derived from Artemisia plants. This derivative was used by the Chinese herbalists for many years to cure several illnesses[2].

In the modern antibiotic era, a new dimension was given to antibiotics by Paul Ehrlich and Alexander Fleming [1]. Ehrlich described antimicrobial as a" magic bullet" which has the ability to target pathogenic microbes alone without disturbing the host. He also proposed that antibiotics synthesize chemical compounds which act mainly on the pathogenic microbes [3]. It was in the year 1928, Sir Alexander Fleming discovered Penicillin. It was derived from fungus *Penicillium notatum* and was widely used to treat many bacterial infections. Fleming cautioned that, usage of Penicillin in inappropriate dosage could result in the development of antibiotic resistance.

Following this path, number of researches were done in the field of medicine resulting in the development of newer antibiotics. The golden period of novel antibiotics discovery was between 1950s and 1970s. Since then no newer class of antibiotics were discovered. Therefore, to prevent the emerging and re-emerging antibiotic resistance newer classes of drugs have to be developed [4].

ANTIBIOTIC RESISTANCE

Antibiotic resistance is defined as the resistance exhibited by a microorganism to an antibiotic to which it was originally susceptible [5]. When this happens, the resistant microorganisms can tolerate the antimicrobials resulting in the failure of effective treatment. This leads to persistence of infections and also increases the risk of transmission. Irrational usage of antibiotics leads to the development of antimicrobial resistance. In other words, simply using antibiotics

leads to the development of resistance . They are meant to be used to manage infections.

CDC says upto 50% of the antibiotics are not correctly prescribed. They are often given when not needed and also in incorrect dosing and duration [6]. Such misuse of antibiotics leads to the emergence of resistance among microorganisms very quickly. Other features accelerating the spread of antibiotic resistance are improper sanitary conditions, food handling practices and inadequate infection control practices[5]. The development of resistant strains is likely when the organisms multiply erroneously. This can also happen when they exchange such resistant traits. A recent data suggests that there are more than 20,000 potential genes of resistance existing in 400 different types [7]. The prospective use of any antibiotic is compromised when resistance develops to that compound right from the first time it is employed.

The term "superbugs" denote microbes with increased mortality and morbidity because of multiple mutations conferring increased levels of resistance. Super resistant strains are those which have acquired enhanced virulence [8]. In short, Antibiotic resistance is a virulence factor. Bacterial resistance to antibiotics is due to chromosomal changes or genetic material exchange through plasmids or transposons [9].

Increasing resistance poses a serious threat to human health which requires immediate corrective action. According to WHO data, an increase in antiretroviral drug resistance has been reported in the year 2013 and 480000 new cases of

MDR-TB were recorded and also extensive drug resistant tuberculosis was identified in 100 countries [5].

Mechanism of resistance to antimicrobials may be Innate or acquired. As per the definitions of EFSA, antimicrobial resistance or susceptibility is defined based on the characters exhibited by the microorganisms in vitro. Such definitions reflect the ability of the bacteria to resist an antimicrobial at a particular concentration, but the definitions vary based on whether the purpose of investigation is for epidemiological survey or for diagnostic purposes [16,17]

Intrinsic Resistance:

It is a characteristic feature of an organism. Reasons may be enzymes produced by bacteria against antimicrobials , inability of certain antibiotics to penetrate the cell membrane of bacteria and in some cases, site of action of the antimicrobial may be masked. Though these bacteria are clinically resistant, they are better referred to as "unsusceptible", as it would turn susceptible by increasing the levels of antimicrobial concentration[19].

Multidrug Resistance

There are multiple definitions and terms used for Multidrug resistance. They are,

- Multidrug Resistant bacteria are the ones which have the capacity to resist various classes of antimicrobials through different modes of action[18] .

- The European Food Safety Association employs the terms Multiple Resistance and Multi-resistance to denote an organism which is resistant to various antibiotics [16,17]
- To denote resistance several terminologies exist namely, Co-resistance, Insusceptibility and Tolerance.
- Insusceptibility is defined as the intrinsic character of an organism like impermeability of cell wall.
- Tolerance is defined as decreased susceptibility exhibited by a bacterium to an antimicrobial characterized by an increase in MIC.
- Resistance can also be transferred to bacteria through genetic elements.

REASONS FOR DEVELOPMENT OF ANTIBIOTIC RESISTANCE

Bacteria turn resistant to antibiotics by several mechanisms like

1)Neutralization of antibiotics

2) Pumping out antibiotics

3) Change in the cellular structure of bacteria which prevents antibiotics from attaching to the cell wall

4)Mutation of their genetic material

After exposure to antibiotics, bacteria can survive by exhibiting one of the above said mechanisms and result in continuous replication. In certain instances, such multiplication can result in serious infections and even death [6]. The critical factor about microbes is that they have the capacity to adapt to any change in

their environment. Irrational usage of antimicrobials has given rise to serious consequences [10]. As of now, majority of microorganisms have turned resistant to many antimicrobials. This could result in the emergence of bacteria causing opportunistic infections. Such infections can result in increased morbidity and mortality rates. In the present scenario, the problem we face is that with increasing discovery of newer antibiotics, the resistance among bacteria is also increasing [11].

Among the genetic changes, Penicillinase enzymes play a significant role in contributing to resistance[12]. Genetic determinants like Transposons also contribute to resistant mechanisms [20]

A bacterium can become resistant by mutation or through acquisition of genes encoding resistance. Genetic elements which are transferred commonly are those which encode enzymes capable of modifying the antimicrobial structure like penicillinases, Cephalosporinases.

Conjugation is a process which transfers plasmids from an organism to another. Resistant genes are carried on elements called Transposons which are not capable of replicating. They have the ability to move inside genetic material. There are elements present on plasmids called integrons which carry several resistant genes [21]. Antibiotics used to manage infections are classified based on their mechanism of action like

- Modifying cell wall structure
- Reducing the synthesis of proteins
- Reducing the synthesis of nucleic acids

- Inhibiting metabolic pathway

Bacteria resist antibiotics by various mechanisms. For example, some bacteria resist antimicrobials by their envelope which limits the penetration by antimicrobials[22,23].

Other mechanisms are

- Bacteria may alter the membrane permeability by decreasing the porin content, altering the LPS structure thereby resulting in reduced antibiotic access to the target site.
- Some bacteria possess efflux pumps that help in extruding the antimicrobial from the cell before it reaches the target site.
- Bacteria may possess plasmids or chromosomal genes that encode enzymes which cleave the antimicrobials before they act.
- Bacteria might acquire mutations which prevent the antimicrobial from reaching the site of action.
- Some bacteria synthesize molecules that mask the target access to antibiotics.
- AcrAB-TolC efflux plays a main role in Enterobacteriaceae. The expression of this efflux pump results in fluoroquinolone resistance. Mutations in gyrase and topoisomerase are the reasons for the development of resistance among *Salmonella* and *Campylobacter* spp [24]
- Similar mechanisms of resistance for macrolides in *Campylobacter* spp have been reported [25]

- Biofilms are another important mechanism exhibited by the bacteria to resist biocides and antibiotics [26,27,28,29]
- Other mechanisms include decreased metabolism, decreased penetration due to extracellular matrix[30] , enzymatic inactivation of biocides [31,32,33] , induction of drug resistant operons and efflux pumps[34]

Resistant mechanisms exhibited by bacteria

Antibiotic resistance in *Salmonella* spp has been related to the presence of Transposons. This picture was seen among MDR *Salmonella* spp[37]. Salmonella Genomic Island-1 contributes to this resistance and its variant Salmonella Genomic Island 1-k also plays an important role in contributing to resistance [38]

Efflux pump contributes to multidrug resistance among *E. coli*[39]. It was shown in a study conducted by Hansen et al, that transfer of gene OqxAB is the reason for reduced susceptibility to various antibiotics like Chloramphenicol and Ciprofloxaicn [40]. The gene mentioned is a mobile genetic element which encodes resistance for biocides and antibiotics. Studies say that *Pseudomonas* spp possess mobile genetic elements[41].

Resistance to Benzalkonium Chloride among Staphylococcal isolates has been reported. Most of the resistant strains exhibited plasmid-borne qac A/B and qac C genes. The association between resistance to the disinfectant and susceptibility to antibiotics was studied in 2002 by Sindhu et al. The study reported that genes encoding resistant mechanisms were the reason behind resistance to antibiotics and disinfectants. It was also found that plasmids contributed to resistant

mechanism in forty four percentage of the isolates. Similar study conducted in 1998 by Paulsen has also proved the association between qac gene and antibiotic resistance among Staphylococcal isolates[36].

Increased antimicrobial usage has resulted in the transfer of mobile genetic elements among bacteria. Some microorganisms act as source of resistant genetic elements accounting for transmission of resistance [19].

BIOCIDES

Biocides are products which destroy or exert a control on pathogenic organisms by chemical or biological means [19]. Large number of biocides are in use. They possess potent antibacterial activity [42].To prevent and control HAIs, appropriate use of biocides is very important.

Biocides can be synthetic or natural derived from bacteria and plants. A biocide can be either a Pesticide like insecticide, fungicide or an antimicrobial like germicide, antibacterial compound.

Appropriate usage of biocidal products play a critical role in infection control protocol[43] . Hospitals are loaded with microorganisms which transmit infectious diseases and also account for constant increase in multidrug resistant organisms. This is a critical alert as such resistant organisms cause infections which are difficult to treat. Infection control program is very essential in all hospitals and in particular, disinfection is a key component of this program. By the appropriate use of disinfectants in correct dilution, many infectious diseases

can be kept under check. Reasons behind increased incidence of MDR bacteria in health care setup are lack of environmental hygiene, inappropriate usage of antimicrobials and transfer of genetic determinants among bacteria.

The effective use of disinfectants in hospitals is essential to prevent HAIs. It was shown in a study, when floorings and surfaces in health care setup are cleaned with Soapwater, the reduction in the microbial load was lesser when compared to cleaning with disinfectants [44]. The fact was proved by another study which showed rise in bacterial load from ten colony forming units per ml to thirty four thousand colony forming units per ml in water used for mopping without adding disinfectant [45]

Disinfectants and Antiseptics are used extensively in hospitals and health care settings to get rid of microorganisms. They are used for a variety of surface applications. They play a key role in controlling infections and preventing nosocomial infections. The widespread use of antiseptic and disinfectant products has provoked some guesses on the development of antimicrobial resistance[46]. A germicide is a one which has cidal effect on microorganisms and it includes both antiseptics and disinfectants.

Antiseptics are those applied to living tissue and skin whereas Disinfectants are applied to inanimate objects[47]. The guidelines of Disinfection and Sterilization should be followed properly to prevent HAIs. There are many studies all over the world which have documented the lack of compliance with these guidelines [48] .

Many disinfectants are used in health-care settings. They are used in combinations (Hydrogen peroxide and peracetic acid) or alone. Commonly used disinfectants include alcohols, chlorine compounds, formaldehyde, glutaraldehyde, iodine compounds, phenols, and quaternary ammonium compounds.

DISINFECTANTS

An ideal disinfectant should be broad spectrum (eliminating bacteria, fungi, viruses, protozoa and spores), nonirritating, nontoxic, noncorrosive and inexpensive. The factors determining the effectiveness of disinfectants are

- Dilution of disinfectants
- Organic matter and Protein content
- Exposure time
- Temperature
- Nature of the bacteria involved
- Extent to which contamination has occurred

Based on the action, disinfectants are classified into three levels namely High, intermediate and low. High level disinfectants kill organisms except for spores, intermediate are the ones which kill Mycobacteria, Viruses and Fungi without action on spores and low level are the ones which are cidal for most vegetative bacteria , certain Fungi and Viruses [49] . As with antibiotics and other

chemotherapeutic drugs, resistance to germicides can also arise by either mutation or acquisition of genes through plasmids or transposons.

Hypochlorites

Hypochlorites are extensively used in hospitals for disinfection. They exhibit an extended spectrum of action on microorganisms, are not affected by hardness of water, cheap and rapid in action, have a low incidence of serious toxicity. They are effective against bacteria, fungi and algae but not spores. To decontaminate hepatitis and HIV viruses, hypochlorites are recommended. Chlorine based disinfectants are inactivated by organic material and blood. Therefore, surfaces must be clean before their use[46].

Mechanism of action

The mode of action of chlorine disinfectants is not proven so far. Chlorine causes disinfection by many factors like enzyme oxidation, amino acids chlorination, intracellular content leakage, reduced nutrient uptake, reduced uptake of oxygen, reduced synthesis of ATP and proteins [50] . The mechanism of action of chlorine is dependent on all these factors.

Quaternary ammonium compounds

This compound's activity is not affected by water hardness. They tolerate residues of anion [51] . They are good cleansers. Their bactericidal action may be reduced when hardness of water increases. Also use of substances like gauze dressing materials and cotton reduce their efficacy by absorption of the activated ingredients [52]. This is shown in a study, where there was a significant decline in

the concentration of compound released (40-50% lower at 1 hour) when cotton materials were used in a wide open container compared to usage of non woven material in a sealed container [53]. Gram negative bacteria can survive in this compound [54]. HAIs have been documented from contaminated solutions used to disinfect devices like cardiac catheters and cystoscopes [55,56].

Mechanism of action

The mechanism of action is through enzyme inactivation, amino acids denaturation. They also disrupt the outer membrane of cell [57,58,59] . They are generally active against fungi, bacteria and viruses which are enveloped. They do not have action on spores [59,60,61,62,63]. These compounds are used in the cleaning of hospital floors, furniture and walls. They are also used to disinfect medical equipments like blood pressure cuffs.

The efficient use of disinfectants is the cornerstone of any infection control program to prevent HAIs. It has been shown in studies that examination of water after mopping the hospital floors showed significant bacterial growth when detergents are used without adding disinfectant. Contamination of such surfaces which are often handled by patients can result in increased exposure to bacteria [64]. It has been shown in investigations that use of soap water to clean hospital surfaces results in increased rate of contamination by bacteria. In a study, using of detergents to clean hospital floors and surfaces resulted in increased bacterial contamination (average increase = 103.6 CFU/24cm^2) [65].

In some conditions when the bacterial contamination from surfaces has not been completely eliminated and when the person cleaning the surface uses the same cloth to wipe some other surface, it results in the transfer of bacteria to that surface and also to the person holding the cloth. CDC protocol suggests, proper disinfection of surfaces should be done when they get contaminated with potential infectious fluids. The protocol also suggests, all materials to which patients are frequently exposed should be disinfected to get rid of certain pathogens like enterococci which have the ability to stay alive in such environment for a longer period [66].OSHA (Occupational Safety and Health Administration) recommends that surfaces contaminated with blood and infectious body fluids must be disinfected.

Contact Time for Surface Disinfectants

The key factor which determines the effectiveness of disinfectant usage is the contact time allowed. The contact time mentioned on the disinfectant product label is not followed in many places the reason being long contact time suggested which is practically not possible. The disinfectant products approved by EPA contain labels which specify contact time of 10 minutes against infectious fluids. But this is not applicable practically as many hospitals allow contact time of less than a minute after disinfectant application. There are many studies which show appreciable reduction in bacterial count when contact time of 30 seconds to 1 minute is allowed [67].

Microbial Contamination of Disinfectants

When disinfectants and antiseptics get contaminated, they serve as vehicles of HAIs. There are many reports describing the fact that contaminated germicides lead to health-care-associated infections [68]. Pseudomonas species frequently contaminate disinfectant solutions. They survive in such contaminated solutions due to their characters like nutritional adaptability, distinct nature of cell membrane which block the disinfectants from entering the cell and efflux pump mechanism [69] . Disinfectants can be contaminated by *Pseudomonas* spp when they are incorrectly diluted [70]. Many studies show that infections arise mainly when contaminated disinfectant solutions are used. Disinfectant products which are more prone for contamination include chlorhexidine, quaternary ammonium compounds and phenolics.

Appropriate actions must be taken in health care set up to prevent the disinfectant products from getting contaminated. The measures to be taken are as follows. First, the disinfectants should be used in appropriate dilution. Second, in health-care setups the sources of contamination of germicides should be tracked and kept under check. Literature says, sources of contamination of disinfectants are the diluents, unhygienic container vessels, and contaminated hospital environment. Stock solutions of disinfectants must be stored as mentioned by the manufacturer [71] .

Factors Influencing The Efficiency Of Disinfectants

To act efficiently against bacteria, disinfectants are dependent on many factors like organism's inherent nature, physical and chemical environment. The time taken by a germicide to destroy microorganisms depends on the number of microbes present. This was shown by Spaulding who conducted experiments and established the fact which says 30 minutes are needed to destroy 10 *Bacillus atrophaeus* spores whereas 3 hours are needed to destroy 10^5 *B.atrophaeus* spores [72]. When bacterial load is reduced and disinfectants are used in correct dilutions, efficiency of the disinfectants increases and also the time required to kill the microbial load gets shortened.

MULTIDRUG RESISTANT BACTERIA:

Treatment of infectious diseases has become more challenging due to increase in the percentage of infections caused by the opportunistic pathogens which are capable of developing resistance to multiple classes of antibiotics.

The above mentioned multidrug resistant bacteria are the well known causes of nosocomial infections especially in ICUs. They have been recorded as the frequently isolated non-fermentative gram negative bacteria by SENTRY Antimicrobial Surveillance Program Medical Centers, 1997-2001 [120]. With these organisms, emergence of resistance during the course of treatment is very commonly noted [121]. In the Global Sentry Antimicrobial Surveillance Program, 1997-1999 the percentage of multidrug resistant *Pseudomonas aeruginosa* recorded was 8.2% in America [122]. In a study done in Brazil, these two organisms

were reported to be the leading multidrug resistant bacilli isolated from patients in ICU [123]. Multidrug resistance among these organisms might be due to administration of empirical antibiotics followed in many hospitals.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is an aerobic, non-fermenting, saprophytic, gram-negative bacilli widely spread in nature, especially in moist environments. This organism secretes a variety of pigments like bluish green pigment pyocyanin, fluorescent , yellowish green pigment pyoverdin, and reddish brown pigment pyorubin. To enhance pigment production, agar mediums are available like King A and King B medium [73] .

P.aeruginosa possess the ability to grow using diesel. So it is also known as Hydrocarbon-using bacteria [74] . *Pseudomonas* is known for its ability to cause opportunistic infections and HAIs. *Pseudomonas* accounts for 9.9% of HAIs[79-82]. Among patients admitted in burns ward [83] *Pseudomonas* outbreak leads to high mortality of upto 60%. In the increasing HIV population, *Pseudomonas* infection causes 50% of deaths. Cystic fibrosis patients are particularly susceptible to chronic infection by *Pseudomonas* with high morbidity and mortality [84]. These overwhelming infections by this organism are contributed by a number of virulence factors like exotoxin A, Proteases, Phospholipase C and Hemolysins [83].

Pseudomonas spp account for 65% death rate in hospitals all over the world [75]. Although they cause disease in healthy individuals, major risk is among hospitalized and immunosuppressed patients. The increase in mortality associated

with these infections is due to combination of weak host defense system and bacterial resistance to antibiotics [76]. Apart from patients' predisposing factors, the risk of *Pseudomonas* infection can be greatly increased by unhygienic hospital environment. Unhygienic hospital environment and unsterilized medical devices are regarded as important sources of infection [77]. As such, contaminated medical devices, catheter tubes, infusions, disinfectants, cleaning liquids have been reported as vehicles of transmission [78].

Pseudomonas causes following HAIs

Pneumonia:

It is an inflammatory condition of the lung affecting the alveoli primarily. Typical symptoms include cough and difficulty in breathing.

Urinary tract infection:

Pseudomonas causes cystitis, pyelonephritis.

Wound infection:

Pseudomonas enters through broken skin causing infection

Blood stream infection:

The organism invades the blood stream resulting in bacteraemia.

Cystic fibrosis:

It is also known as mucoviscidosis. It affects various organs like pancreas, liver, kidneys, and intestine but mainly it affects lungs.

Pseudomonas aeruginosa is a major threat to patients admitted in ICUs. Data obtained from the National Nosocomial Infections Surveillance System says, *P.aeruginosa* causes significant percentage of Pneumonias, UTIs, and ENT infections among patients admitted in Intensive care units [89]. In a surveillance study, *Pseudomonas aeruginosa* accounted for thirty percent of pneumonias, nineteen percent of urinary tract infections, and ten percent of bloodstream infections[90].

Pseudomonas aeruginosa is gaining importance clinically due to its high resistance to antimicrobial agents. It causes opportunistic infections among humans. Resistance of *pseudomonas* to antibiotics and disinfectants is an increasing problem in healthcare setting. Though rarely it occurs as a normal flora in humans, it is frequently isolated from immunosuppressed patients and from patients associated with burns, cystic fibrosis and neutropenia[91].

Pseudomonas aeruginosa shows innate resistance to many disinfectants and antibiotics. The antimicrobial agents used against *Pseudomonas aeruginosa* are Fluoroquinolones, Aminoglycosides, Antipseudomonal penicillins and Cephalosporins. However, an alarming increase in resistance to various antimicrobial agents have been reported both from India and abroad [83,84,85-88]. Currently *Pseudomonas* is showing increased resistance to various antibiotics like quinolones, aminoglycosides and carbapenems. The mechanisms of resistance to antibiotics include reduction in cellwall permeability, production of betalactamases which are both chromosomal and plasmid mediated, production of

aminoglycoside-modifying enzymes and an active multidrug efflux mechanism[92]. Nalidixic acid was the first quinolone used to treat urinary system infections caused by *Pseudomonas* but it has a narrow spectrum of action with high incidence of resistance. In late 1980s, it has been discovered that addition of fluorine atom and piperazine ring to its structure results in widening of the spectrum. However, *Pseudomonas* has shown resistance to such antimicrobial agents[93]. Because of the evolution of multidrug resistant *Pseudomonas* in ICU resulting in increased morbidity and mortality, empirical usage of antibiotics has been restricted in order to keep the resistance development under check [94]

Resistant pattern exhibited by *Pseudomonas aeruginosa* strains to Fluoroquinolones was examined in a study [95]. It was found that *Pseudomonas* isolates from patients admitted in hospital were more resistant to the mentioned antimicrobial and in particular, those from ICU showed increased resistance to the same. The main mechanism for the development of resistance to fluoroquinolones is the change caused in DNA gyrase enzyme which decreases the binding of the target quinolones to the enzymes. The other mechanisms are the porin channel defect which reduces the amount of quinolones entering the cells and the efflux pump mechanism, located in the membrane, which pump the drug out of the bacteria[96].

Pseudomonas spp exhibit resistance to Imipenem as well. CLSI guideline has changed the MICs of piperacillin, piperacillin tazobactam, imipenem and meropenem which are used to treat *Pseudomonas* infections [97]

In a study conducted in Iran, effects of ciprofloxacin and ceftazidime on *P.aeruginosa* were evaluated. This study showed percentage of isolates sensitive to Ciprofloxacin and Ceftazidime was sixty nine and fifty nine respectively [98]. This organism is an important opportunistic pathogen with innate resistance to many antibiotics. In a study conducted by Bonaventura, sensitivity of *Pseudomonas* was 81.2% for ceftazidime, 93.7% for ciprofloxacin and 88.7% for imipenem[99]. In a study by Kelly et al, sensitivity results of *pseudomonas* was 95% for cephalosporin[100]

Pseudomonas aeruginosa can contaminate many disinfectants like Chlorhexidine and Benzalkonium Chloride which reduces the ability of the disinfectant to reduce the bacterial growth. These disinfectants destroy the outer membrane of cell leading to leakage of intracellular contents and finally lysis of bacteria [101]. When disinfectants are used in below optimum concentrations, it results in the induction of efflux pump mechanism [102]. This process depends on the factor AlgU [103].

Disinfectants up-regulate and down-regulate many genes. For example, after exposure to chlorhexidine, *oprH-phoPQ* and *mexCD-oprJ* operons are up-regulated and genes which encode proteins necessary for functions like transport of electrons, repair of DNA are downregulated [104].

Tolerance to disinfectants among *Pseudomonas* spp is due to formation of biofilms [105].

Acinetobacter baumannii

A Dutch microbiologist Martinus Willem Beigerinck, in the year 1911, discovered the microbe *Acinetobacter* which is a gram-negative, non-fermentative bacterium. It grows under aerobic condition [106]. In the late 1970s, *Acinetobacter* bagan to be recognized as a significant hospital pathogen and it responded to the common antibiotics.

Multidrug resistant *Acinetobacter* causes wide range of infections. It accounts for increased morbidity and mortality rates [107,108]. Potential sources of *Acinetobacter baumannii* include hospital floors, tabletops, pillows, mattresses and sinks[109]. Sources of habitat for *Acinetobacter* include food, arthropods, animals and humans apart from main sources like moist soil and water.

Acinetobacter causes infections mainly among patients admitted in Intensive care units, patients on ventilators, and those infected with burns [110,111]. *Acinetobacter* spp mainly affect immunosuppressed persons and those with underlying chronic diseases [111]. As an opportunistic pathogen, *Acinetobacter* usually possess no threat to healthy people. It is a skin colonizer among normal individuals[109,110]. There are many studies which say, *Acinetobacter* spp cause infections of wound, bone, and surgical sites [112].

As per report released by CDC in the year 2006, steps to prevent *Acinetobacter* infections are educating staff nurses, maintaining personal hygiene, following precautions before handling patients and improving hospital environmental hygiene. The increasing number of such infections and the

multidrug resistant picture exhibited by these isolates stress the necessity of infection control practices to be followed in hospitals [113].

If proper precautions are followed, *Acinetobacter* infections can be kept under control. There are many studies stating that control measures when followed properly decrease the incidence of MDR pathogens [114].

Antibiotic Susceptibility testing

Susceptibility testing to antibiotics is performed on bacterial isolates in clinical laboratories. Following the discovery of penicillin in the year 1928 by Alexander Fleming, many antibiotic compounds were discovered to treat infections[106]. But resistant mechanisms exhibited by the bacteria towards antimicrobials resulted in a different scenario. It became essential to test bacteria isolated from patients against different antibiotic dilutions to establish the antibiotic susceptibility pattern. Broth dilution was the method followed in the earlier days [115,116]. Though broth dilution methods are standard procedures, they consume much time which resulted in the discovery of diffusion method on agar plate by using impregnation discs.

Antibiotic susceptibility can be measured both qualitatively and quantitatively. The qualitative method includes the most widely used diffusion technique Kirby-Bauer disc diffusion test and stokes disc diffusion test. The quantitative method measures the exact concentration of the drug at which the organism is inhibited or killed. This method includes the tube dilution test and agar dilution test.

Researchers started using Agar dilution method to determine the MICs of antimicrobials against bacteria. To determine the efficacy of antimicrobials or to determine the MIC of antibiotics, dilution methods are used frequently[117]

Principle

At the beginning of the century, Rideal, Walker and others proposed the principle of determining the effectivity of an antimicrobial to an organism. The increase in invention of new antimicrobials made these tests and their modification burdensome since large numbers of tests were necessary in daily routine. Alexander Fleming discovered ditch plate method of Agar diffusion and this stood as the forerunner for subsequent inventions.

In laboratories, antimicrobial sensitivity testing was carried out daily after discovery of many antibiotics. Lawn culture of the selected bacterium was made in an agar plate and discs impregnated with the drug were placed on the agar. The impregnated drug diffuses into the agar. Discs are the most common ones used as drug carriers in laboratories though many sources are available.

Though Automation methods have taken an upper hand, these simple techniques are easy to perform and followed in many labs. In the present scenario, Microbiologists should learn the principle of all the available tests, to analyze their benefits and pitfalls to carry-out the ideal one in laboratories. It is also essential to update the recent advances now and then. The methods used to determine the susceptibility or resistant pattern of an antimicrobial uses any one of

the two principles namely Diffusion or Dilution (Agar/Broth). Automated methodology is also based on any one of the above mentioned principles [118].

Dilution Test

MIC test is used to determine the least concentration of a drug which hampers the detectable growth of an organism . Antimicrobial selected is diluted serially in Agar or Broth following which test organism is inoculated serially in prepared dilutions. They are incubated overnight following which minimal inhibitory concentration of the drug can be determined.

MIC tests are costlier and time consuming than diffusion tests but the advantage is, they provide valuable information required to treat patients not responding to antimicrobial therapy.

MBC is used to determine the cidal action of an antibiotic. It is the least amount of a drug which is necessary to destroy an organism. To know whether the drug has killed or inhibited the bacteria, subcultures are done from the test dilutions on to a freshly prepared drug free medium and incubated for 18-24 hours.

The gold standard method of susceptibility testing is Agar dilution method. It is also considered as the most accurate method to determine the MIC. It is easy to perform and also cheaper when compared to other methods. Also upto thirty samples can be tested at a time and so agar dilution is useful for batch tests [115]

Antibiotic Susceptibility characters of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is known for its multidrug resistance. It is one of the common resistant organisms encountered in clinical laboratories. Literature says *Pseudomonas* species are "naturally" resistant as they show resistance to antibiotics administered for first time. Due to this factor, infection with this organism is not treated with a single agent. Normally, Penicillins like Ticarcillin, Mezlocillin or Piperacillin are used in combination with an Aminoglycoside like Gentamicin, Tobramycin or Amikacin. Other drugs which are active against *Pseudomonas* are the newer Quinolones like Ciprofloxacin, Monobactam like Aztreonam, Imipenem and 3rd generation Cephalosporins.

It should be noted that susceptibility pattern exhibited by the organism varies geographically. In the present scenario, around twenty five percentage of the organism isolated is reported to be resistant to all Fluoroquinolones. Plasmids and mutations also have a significant influence on resistance development.

Among patients infected with burns wound, prevention of colonization by *Pseudomonas* is practically not possible. Bacterial load should be maintained below 10^5 to reduce the complications. This can be achieved by the topical application of silver sulfadiazine cream.

Among *Pseudomonas cepacia*, most strains are susceptible to Chloramphenicol, and Co-trimoxazole. In some cases, they also respond to Ceftazidime. They are resistant to β-lactams, aminoglycosides and fluoroquinolones.

Pseudomonas pseudomallei respond to Tetracycline, Co-trimoxazole, Chloramphenicol and Sulfonamides. For better response, drugs are given in combination and administered atleast for 8 weeks. To treat extrapulmonary lesions, 6 months to 1 year of treatment is needed. They show resistance to broad-spectrum Cephalosporins and many aminoglycosides. *Pseudomonas mallei* infections are treated with combination of Aminoglycoside and Tetracycline.

Antibiotic Susceptibility Characters of *Acinetobacter* spp

Resistance to antibiotics is very commonly seen with *Acinetobacter* spp. They are resistant to drugs like Penicillins, Cephalosporins and Chloramphenicol. Resistance to aminoglycosides is also noted in some species. The antibiotics effective against *Acinetobacter* are Aminoglycosides like Gentamicin, Amikacin, Tobramycin , newer Penicillins and Cephalosporins. They are susceptible to Carbapenems like Imipenem but resistance to these drugs are emerging slowly. Antibiotic susceptibility testing should be performed to choose the correct antimicrobial agent [119]

Susceptibility of bacteria to herbal extract

Medicinal plants are an essential part of human society to fight diseases, from the dawn of civilization. In this study, antibacterial effect of neem leaves has been explored. The neem tree was described as *Azadirachta indica* by De Jussieu in early 1830s and so it is known as *Azadirachta indica* A. Juss. It belongs to the Meliaceae family [125] . In taxonomic position, it belongs to the order Rutales but recent literature says that it belongs to the order Sapindales [132] as shown below

Kingdom	**Plantae**
Division	**Magnoliophyta**
Class	**Magnoliopsida**
Order	**Sapindales**
Family	**Meliaceae**
Genus	***Azadirachta***
Species	***A. indica***
Scientific Name	***Azadirachta indica***

Neem trees are widely popular in our country since many years . Neem is known for its ability to cure many pathogenic diseases. Neem is one of the most extensively researched herb and various parts of the tree have a variety of applications [124]. Parts of Neem tree are known to have a broad range of antimicrobial activity. It grows in India , Tropical and Semi tropical areas. They grow very fast and can reach even 15 to 20 meters height. *A. indica* is known by different names like Sacred Tree, Heal all, Village Pharmacy and Panacea for many ailments.

Parts of Neem tree yield many products which possess unique medicinal property. In India, twigs of Neem tree are advised to be used as chewing sticks in traditional medicine[126].

Different parts of Neem tree are known to exhibit various properties like antihelminthic, anti-fungal, anti-diabetic, antibacterial, contraceptive and sedative[125]. It is also known to cure skin diseases. Other functions include healthy hair, improvement in liver function, detoxifying the blood, Pest and disease control, treatment of dental ailments, chronic cough, bronchial asthma, ulcers, piles, intestinal worms, and urinary diseases. They act against many viral diseases [132].

A. indica is a traditional medicine for management of Diabetes and it has a potent anti-diabetic activity [130]. Neem leaf extract acts as a good anti-hyperglycemic agent in both IDDM and NIDDM [131].

The aqueous extract of neem bark shows immunological activity as well. They show anti-complement activity, acting on both the pathways of complement activation in human serum[127]. The leaf extract also stimulate the immune system strongly both by humoral and cell-mediated responses[128]. It has been shown in a study, that oral administration of extract from Neem leaves increases the antibody levels in serum [129].

They reduce cholesterol levels and also improve the functioning of Liver enzymes[133]. In a study, it was reported that certain components present in Neem leaf account for activity against bacteria and fungi[134].

In another study, antibacterial effect of Neem leaf extract was compared with Gentamicin. In a study, it has been proposed that, aqueous extract of Neem leaves can reduce aqueous silver ions to stable nanoparticles of silver in water. Thus there would be synergistic action of antibacterial effect of Neem leaves to the biosynthesized nanoparticles [135].

4. Materials and Methods

Study Period

The Study was done during the period January 2015 to July 2016.

Study Type

Prospective study.

Sample size:

50 isolates each of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were included.

Study Population

Samples were collected from our Microbiology laboratory received from inpatients and outpatients attending PSG Hospitals.

Ethics Approval

Approval was obtained from the Institutional Ethics Committee at the start of the study and was renewed periodically during the study period.

Antimicrobial agents

Antimicrobial agents used in this study are Gentamicin, Ciprofloxacin, and Imipenem obtained from commercial source (Sigma pharmaceuticals).

Disinfectant

Disinfectants chosen for this study are commercially available 3% Sodium Hypochlorite and 4% Benzalkonium chloride (Microlyse)

These disinfectants are commonly used in healthcare setup for surface disinfection. It is mandatory to use them in appropriate concentration to prevent the emergence of multidrug resistant organisms.

Bacterial Isolates

Bacterial isolates used in this study are *Pseudomonas aeruginosa* (50 in number) and *Acinetobacter baumannii* (50 in number). These isolates are obtained from various samples received in our Microbiology Laboratory.

Collected isolates were stored in semisolid agar and renewed during processing.

Herbal Extract

Herb chosen for this study is neem leaves *(Azadirachta indica)*. Botanical Survey of India, Southern Regional Centre, Coimbatore identified and authenticated the collected leaves. The plant specimen is identified as *Azadirachta indica* A.juss.-MELIACEAE.

Culture Media

Culture media used in this study and their composition are

Mueller-Hinton Agar

Beef extract- 2 gm

Acidicase Peptone- 7.5 gm

Starch – 1.5 gm

Agar- 17 gm

Distilled water- 1000 ml

Nutrient Agar

Agar powder – 1.5 to 1.8 gm

Nutrient broth – 100 ml

Nutrient Broth

Peptone – 1 gm

Beef extract – 0.3 gm

Sodium chloride – 0.5 gm

Distilled water – 100 ml

METHODS

1) To determine the MIC of Gentamicin, Ciprofloxacin and Imipenem to *Pseudomonas aeruginosa* and *Acinetobacter baumannii* by agar dilution method.
2) To expose the above bacterial isolates to varying concentrations of Sodium hypochlorite and Benzalkonium chloride.
3) To determine the MIC of Gentamicin, Ciprofloxacin and Imipenem to the bacterial isolates after treatment with the above disinfectants.
4) To prepare extract from neem leaves.
5) To find out the susceptibility of the bacterial isolates to the prepared neem leaf extract before and after exposure to disinfectants.

MIC is the lowest amount of a drug required to hamper the macroscopic growth of an organism after overnight incubation. Agar dilution method is used to determine the MIC in this study.

Inoculum Preparation:

Standardization of inoculum is critical to produce reproducible MICs. Inoculum should match McFarland 0.5 turbidity standard which corresponds to working inoculum of 10^7 CFU/ml.

Preparation of Stock solution

Formula used to prepare Stock solution is

$$1000 \times V \times C \div P = W$$

P = Potency

V = Volume in ml

C = End concentration of solution per ml

W = Weight of the drug to be dissolved

To make 10 ml solution of strength 10,000 mg/L from a powder with potency 980 µg / mg, the quantity of drug required is

$$W = 1000 \times 10 \times 10 \div 980$$

$$= 102.04 \text{ mg}$$

Agar dilution method

- Mueller Hinton agar is prepared, sterilized, distributed in test tubes in exact aliquots sufficient to dilute the desired antimicrobial concentration tenfold.
- Appropriate volume of antimicrobial agent is added in each tube, contents are mixed by gentle inversion, poured into Petri dishes which are sterile and allowed for solidification.
- For growth control, plates containing drug free agar is prepared.

Calculation

Final volume of medium in plate = 20 ml

Top antibiotic concentration = 64 mg/L

Total amount of drug per plate = 1280 µg/ml

2 ml of 1280 µg/ml will be required to start the dilutions

2560 µg /2 ml = 1.28 ml of 2000 µg/ml + 0.72 ml of water

Stock dilution of 2000 µg/ml will be required for this range of MIC.

Organisms are diluted by inoculating 50 µl from an overnight broth culture into 5ml of peptone water to a suspension of approximately 10^7 CFU/ml.

1µl of 10^7 CFU/ml is delivered to the agar surface resulting in a final desired inoculum of 10^4 CFU per spot (should be done within half hour of preparation of dilution).

Inoculated plates are incubated overnight at 37°C

The least amount of drug required to hamper the macroscopic growth is the MIC.

Exposure of bacterial isolates to disinfectants

- The bacterial isolates were inoculated in 10 ml of nutrient broth and incubated overnight.

- Following this, 0.1 ml of inoculums from the broth were inoculated in a series of tubes containing ten ml of freshly prepared nutrient broth containing different concentrations of disinfectants namely, Sodium hypochlorite (3%, 2%,

1%, 0.5%, 0.25%, 0.125%, 0.06%, 0.03%) and Benzalkonium chloride (4%, 3%, 2%, 1%, 0.5%, 0.25%, 0.125%, 0.06%).

-All the tubes were incubated for 1to 2 days at 37°c

-The tubes were checked for turbidity by comparing with the control tube without disinfectant.

-The lowest concentration of disinfectant which inhibits the macroscopic growth of the organism is the MIC.

- Highest concentration of disinfectants allowing bacterial growth were noted. From those tubes,0.1 ml of samples were taken and streaked on nutrient agar plates. Then they were incubated overnight at 37°c to score the survivor colonies.

-MIC of these colonies to Gentamicin, Ciprofloxacin and Imipenem were determined by Agar dilution method as described above.

EFFECT OF HERBAL EXTRACT ON BACTERIAL ISOLATES

PREPARATION OF NEEM LEAF EXTRACT[176]

Neem leaves were collected, shade dried and ground coarsely. Approximately 30 grams or a handful of ground powder was mixed with 100 ml of ethanol. It was kept at room temperature for 72 hours and then filtered by means of Whatman No.1 filter paper. The filtered product was then kept in a waterbath for evaporation, till completely dry. The product was mixed with a small amount of 50% ethanol, and used as extract of 100% concentration. It was stored at 4°C, till the time of usage. The subsequent concentrations of neem extract were obtained by the addition of 50% ethanol to the concentrate, in required quantities.

SUSCEPTIBILITY TO PLANT EXTRACT[176]

Susceptibility to neem extract was tested by disc diffusion method. Lawn culture of the isolate was made on Mueller Hinton agar plate. Four sterile discs were placed and neem extract was added to the discs in five different concentrations 5%, 10%, 15%, and 25% . The volume added to each disc was 20µl. The plate was incubated at 37°C overnight. If the bacterial isolate is susceptible to neem leaf extract, uniformly circular zone of inhibition will be formed.

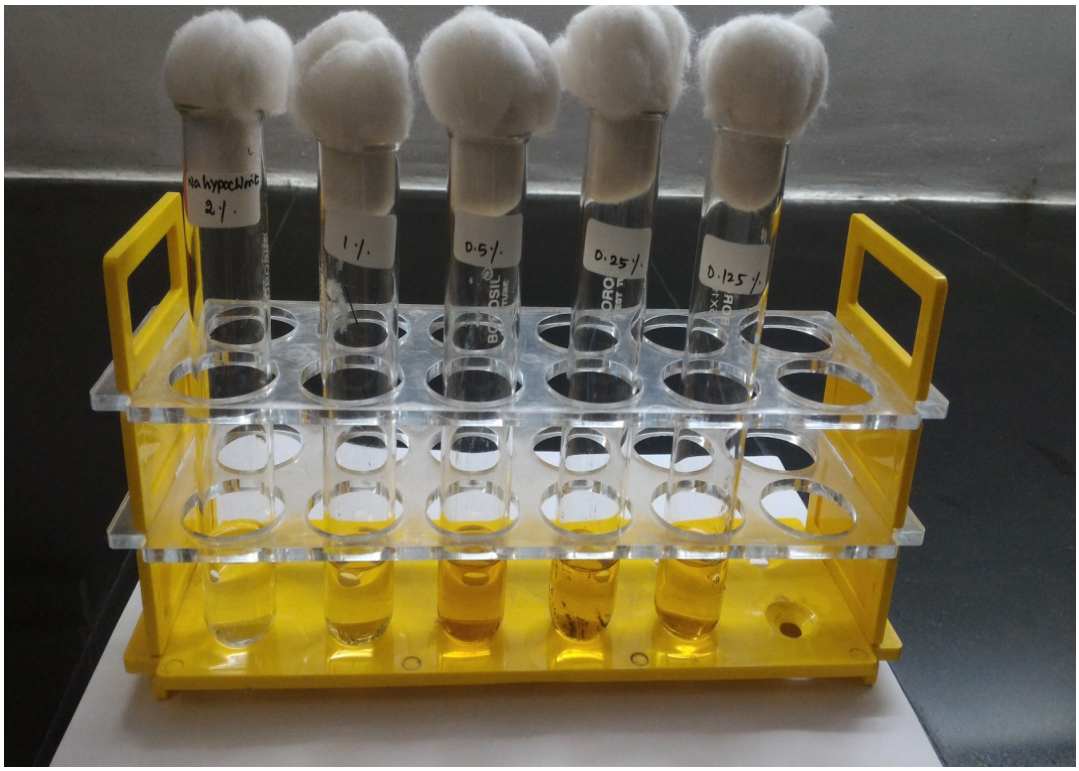
5. Results

In this study done for a period of nineteen months (January 2015 to July 2016), the selected bacteria *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (50 isolates each) received in our microbiology laboratory were exposed to traditional disinfectants used in hospitals, Sodium Hypochlorite and Benzalkonium Chloride, following which the MIC of antibiotics Ciprofloxacin, Gentamicin and Imipenem to the isolates were determined and was compared with their MIC before exposure. The susceptibility of the selected isolates to the prepared Neem leaf extract before and after disinfectant exposure was also determined. The results are as follows.

EXPOSURE OF BACTERIA TO DISINFECTANTS

Isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were exposed to varying concentrations of 3% Sodium Hypochlorite ranging from 3% to 0.03%. Growth was observed in 0.25%, 0.125%, 0.06 %and 0.03% for these isolates. Similarly they were exposed to varying concentrations of 4% Benzalkonium chloride ranging from 4% to 0.06%. Growth was observed in 1%, 0.5%, 0.25%, 0.125% and 0.06% for *Pseudomonas* isolates and in 0.5%, 0.25% and 0.125% and 0.06% for *Acinetobacter* isolates.

Picture 1 – Exposure of *Pseudomonas aeruginosa* isolates to varying concentrations of Sodium Hypochlorite



Picture 2- Exposure of *Acinetobacter baumannii* isolates to varying concentrations of Benzalkonium chloride (Microlyse)

MIC OF ANTIBIOTICS TO THE SELECTED ISOLATES

Out of 50 isolates of *Pseudomonas aeruginosa*, number of isolates sensitive to Ciprofloxacin, Gentamicin and Imipenem were 36(72%), 31(62%) and 46(92%) respectively. The range of MIC for Ciprofloxacin, Gentamicin and Imipenem were 0.06 to 8 µg/ml, 0.5 to 128 µg/ml and 0.25 to 16 µg/ml respectively (Table 1)

Table 1- MIC of Antibiotics against *Pseudomonas aeruginosa* (50 Isolates)

MIC Dilution of drug (µg/ml)	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**	**0.25**	**0.125**	**0.06**
No of Isolates **Ciprofloxacin**	-	-	-	-	3	11	0	5	3	12	8	8
No of Isolates **Gentamicin**	7	8	4	-	-	10	8	11	2	-	-	-
No of Isolates **Imipenem**	-	-	-	1	1	2	13	13	17	3	-	-

Numbers underlined indicate resistance

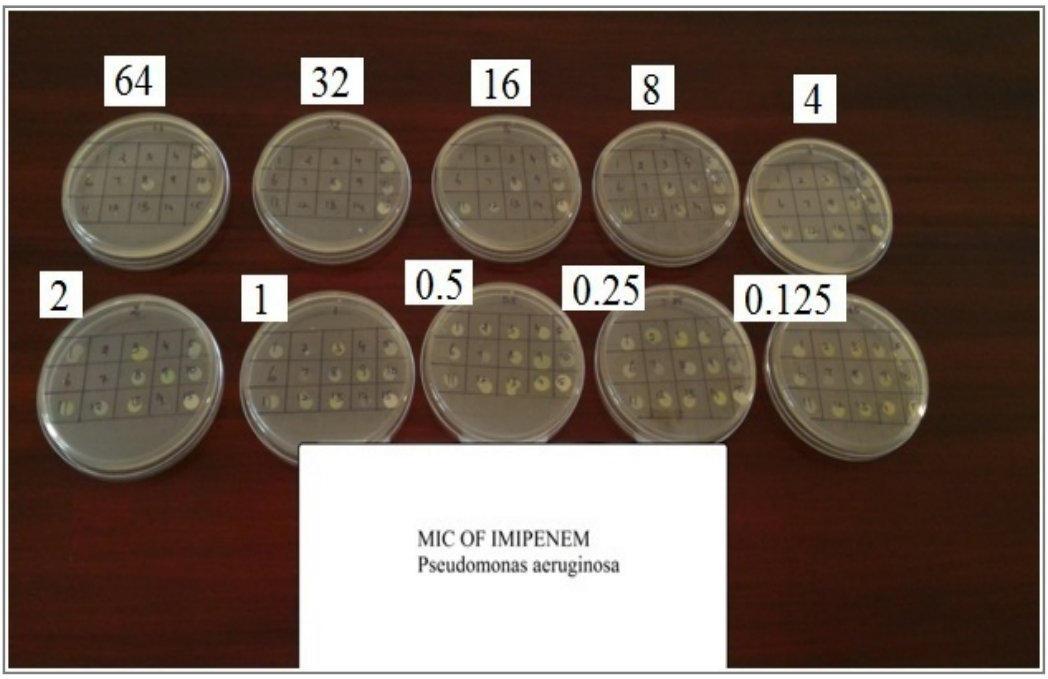
Ciprofloxacin – Sensitive ≤ 1 ; Intermediately sensitive 2 ; **Resistant ≥ 4**

Gentamicin - Sensitive ≤ 4 ; Intermediately sensitive 8 ; **Resistant ≥ 16**

Imipenem – Sensitive ≤ 2 ; Intermediately sensitive 4 ; **Resistant ≥ 8**

Picture 3 – MIC of Imipenem on plates of Mueller Hinton Agar by Agar Dilution method

(Concentration of drug in µg/ml indicated below)



Out of 50 isolates of *Acinetobacter baumannii*, the number of isolates sensitive to Ciprofloxacin, Gentamicin and Imipenem were 33 (66%), 33(66%) and 43(86%) respectively. The range of MIC for Ciprofloxacin, Gentamicin and Imipenem were 0.06 to 32 µg/ml, 0.5 to 128 µg/ml and 0.03 to 16 µg/ml respectively. (Table 2)

Table 2- MIC of Antibiotics against *Acinetobacter baumannii* (50 Isolates)

MIC **Dilution of drug** (µg/ml)	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**	**0.25**	**0.125**	**0.06**	**0.03**
No of Isolates **Ciprofloxacin**	-	-	1	2	2	5	7	14	11	4	3	1	-
No of Isolates **Gentamicin**	2	3	1	4	7	10	10	9	4	-	-	-	-
No of Isolates **Imipenem**	-	-	-	1	2	4	11	10	6	3	3	5	5

Numbers underlined indicate resistance

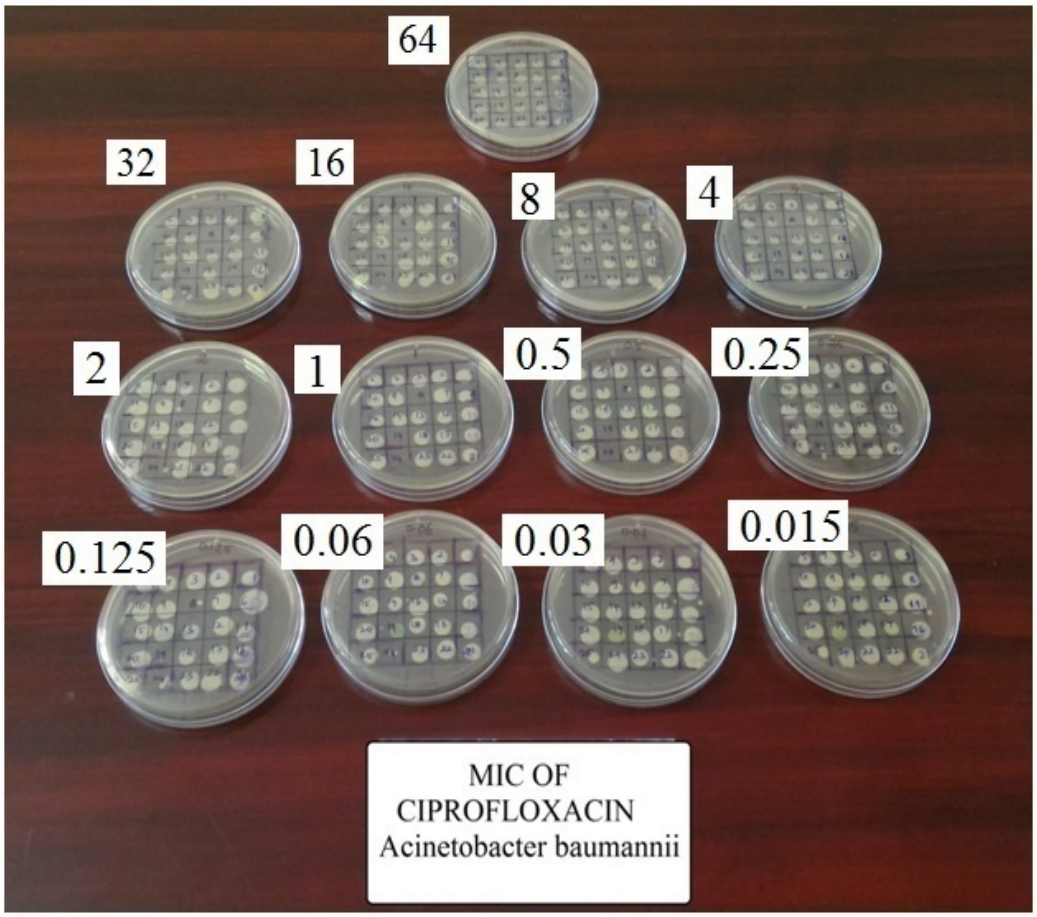
Ciprofloxacin – Sensitive ≤1 ; Intermediately sensitive 2 ; **Resistant ≥ 4**

Gentamicin - Sensitive ≤4 ; Intermediately sensitive 8 ; **Resistant ≥ 16**

Imipenem – Sensitive ≤ 2; Intermediately sensitive 4 ; **Resistant ≥ 8**

Picture 4- MIC of Ciprofloxacin on plates of Mueller Hinton Agar by Agar dilution method

(Concentration of drug in µg/ml indicated below)



MIC OF ANTIBIOTICS TO THE SELECTED ISOLATES AFTER EXPOSURE TO DISINFECTANTS

The number of isolates of *Pseudomonas aeruginosa* sensitive to Ciprofloxacin, Gentamicin and Imipenem were 27(54%), 21(42%) and 28(56%) respectively. The resistant pattern was 22 (44%), 28(56%) and 14(28%) to Ciprofloxacin, Gentamicin and Imipenem respectively. The range of MIC for Ciprofloxacin, Gentamicin and Imipenem were 0.06 to 32 µg/ml, 1 to 128 µg/ml and 0.5 to 16 µg/ml respectively. (Table 3)

Table 3- MIC of antibiotics to *Pseudomonas aeruginosa* after exposure to Sodium Hypochlorite (50 Isolates)

MIC **Dilution of drug** (µg/ml)	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**	**0.25**	**0.125**	**0.06**
No of Isolates **Ciprofloxacin**	-	-	2	2	5	13	1	4	10	6	5	2
No of Isolates **Gentamicin**	10	7	3	8	1	5	11	5	-	-	-	-
No of Isolates **Imipenem**	-	-	-	3	11	8	15	12	1	-	-	-

Numbers underlined indicate resistance

Ciprofloxacin – Sensitive ≤ 1 ; Intermediately sensitive 2 ; **Resistant ≥ 4**

Gentamicin - Sensitive ≤ 4 ; Intermediately sensitive 8 ; **Resistant ≥ 16**

Imipenem – Sensitive ≤ 2 ; Intermediately sensitive 4 ; **Resistant ≥ 8**

After exposure to highest concentration of Benzalkonium chloride which allowed bacterial growth, the number of sensitive isolates were 19(38%), 8(16%) and 19(38%) for Ciprofloxacin, Gentamicin and Imipenem respectively. The number of isolates resistant to Ciprofloxacin, Gentamicin and Imipenem were 28(56%), 34(68%) and 23(46%) respectively. The range of MIC for Ciprofloxacin, Gentamicin and Imipenem were 0.06 to 16 µg/ml, 1 to 128 µg/ml and 0.5 to 16 µg/ml respectively. (Table 4)

Table 4- MIC of Antibiotics to *Pseudomonas aeruginosa* after exposure to Benzalkonium chloride (50 Isolates)

MIC **Dilution of drug** (µg/ml)	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**	**0.25**	**0.125**	**0.06**
No of Isolates **Ciprofloxacin**	-	-	-	1	10	17	3	15	3	-	-	1
No of Isolates **Gentamicin**	10	8	2	14	8	6	1	1	-	-	-	-
No of Isolates **Imipenem**	-	-	-	5	18	8	6	10	3	-	-	-

Numbers underlined indicate resistance

Ciprofloxacin – Sensitive ≤ 1 ; Intermediately sensitive 2 ; **Resistant ≥ 4**

Gentamicin - Sensitive ≤ 4 ; Intermediately sensitive 8 ; **Resistant ≥ 16**

Imipenem – Sensitive ≤ 2 ; Intermediately sensitive 4 ; **Resistant ≥ 8**

After exposure to highest concentration Sodium hypochlorite which allowed bacterial growth, the number of isolates of *Acinetobacter baumannii* sensitive to Ciprofloxacin, Gentamicin and Imipenem were 17(34%), 21(42%) and 28(56%) respectively. The resistant pattern was 21(42%), 24(48%) and 17(34%) to Ciprofloxacin, Gentamicin and Imipenem respectively. The range of MIC for Ciprofloxacin, Gentamicin and Imipenem were 0.25 to 32 µg/ml,1 to 128 µg/ml, and 0.03 to 32 µg/ml respectively. (Table 5)

Table 5 – MIC of Antibiotics to *Acinetobacter baumannii* after exposure to Sodium Hypochlorite (50 Isolates)

MIC **Dilution of drug** (µg/ml)	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**	**0.25**	**0.125**	**0.06**	**0.03**
No of Isolates **Ciprofloxacin**	-	-	2	2	8	9	12	8	8	1	-	-	-
No of Isolates **Gentamicin**	3	2	6	13	5	11	5	5	-	-	-	-	-
No of Isolates **Imipenem**	-	-	1	6	10	5	13	9	4	-	-	1	1

Numbers underlined indicate resistance

Ciprofloxacin – Sensitive ≤ 1 ; Intermediately sensitive 2 ; **Resistant ≥ 4**

Gentamicin - Sensitive ≤ 4 ; Intermediately sensitive 8 ; **Resistant ≥ 16**

Imipenem – Sensitive ≤ 2 ; Intermediately sensitive 4 ; **Resistant ≥ 8**

After exposure to highest concentration of Benzalkonium chloride which allowed bacterial growth, the number of *Acinetobacter baumannii* isolates sensitive to Ciprofloxacin, Gentamicin and Imipenem were 18(36%), 8(16%) and 23(46%) respectively. The number of resistant isolates to Ciprofloxacin, Gentamicin and Imipenem were 25(50%), 34(68%) and 20(40%) respectively. The range of MIC for Ciprofloxacin, Gentamicin and Imipenem were 0.5 to 32 µg/ml , 1 to 128 µg/ml and 0.06 to 16 µg/ml respectively (Table 6)

Table 6-MIC of Antibiotics to *Acinetobacter baumannii* after exposure to Benzalkonium chloride(50 Isolates)

MIC **Dilution of drug**(µg/ml)	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**	**0.25**	**0.125**	**0.06**
No of Isolates **Ciprofloxacin**	-	-	1	3	13	8	7	16	2	-	-	-
No of Isolates **Gentamicin**	10	8	2	14	8	6	1	1	-	-	-	-
No of Isolates **Imipenem**	-	-	-	4	16	7	3	13	3	1	1	2

Numbers underlined indicate resistance

Ciprofloxacin – Sensitive ≤ 1 ; Intermediately sensitive 2 ; **Resistant ≥ 4**

Gentamicin - Sensitive ≤ 4 ; Intermediately sensitive 8 ; **Resistant ≥ 16**

Imipenem – Sensitive ≤ 2 ; Intermediately sensitive 4 ; **Resistant ≥ 8**

Comparison of resistance pattern exhibited by the isolates before and after exposure:

The resistance pattern exhibited by the isolates of *Pseudomonas aeruginosa* increased after treating with disinfectants (Table 7). The percentage of resistance developed after exposure to Benzalkonium Chloride was more when compared to that of Sodium Hypochlorite.

Table 7– Comparison of Resistance pattern to Antibiotics exhibited by *Pseudomonas aeruginosa* before and after exposure to Disinfectants

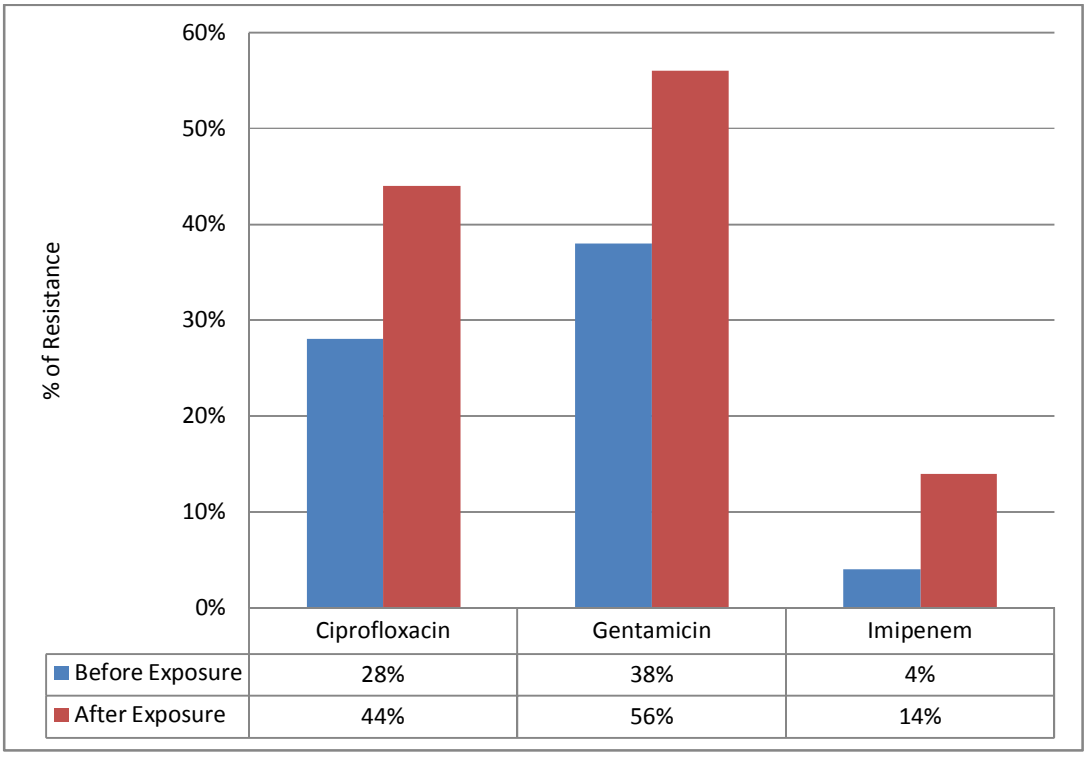
Antimicrobial agents	Before exposure to disinfectants	After exposure to disinfectant (Sodium hypochlorite)		After exposure to disinfectant (Benzalkonium chloride)	
	% of resistance	% of resistance	Increase in resistance	% of resistance	Increase in resistance
Ciprofloxacin	28%	44%	16%	56%	28%
Gentamicin	38%	56%	18%	68%	30%
Imipenem	4%	14%	10%	20%	16%

The resistance pattern exhibited by the isolates of *Acinetobacter baumannii* increased after treating with disinfectants (Table 8). The percentage of resistance developed after exposure to Benzalkonium Chloride was more when compared to that of Sodium Hypochlorite.

Table 8– Comparison of resistance pattern to Antibiotics exhibited by *Acinetobacter baumannii* before and after exposure to disinfectants

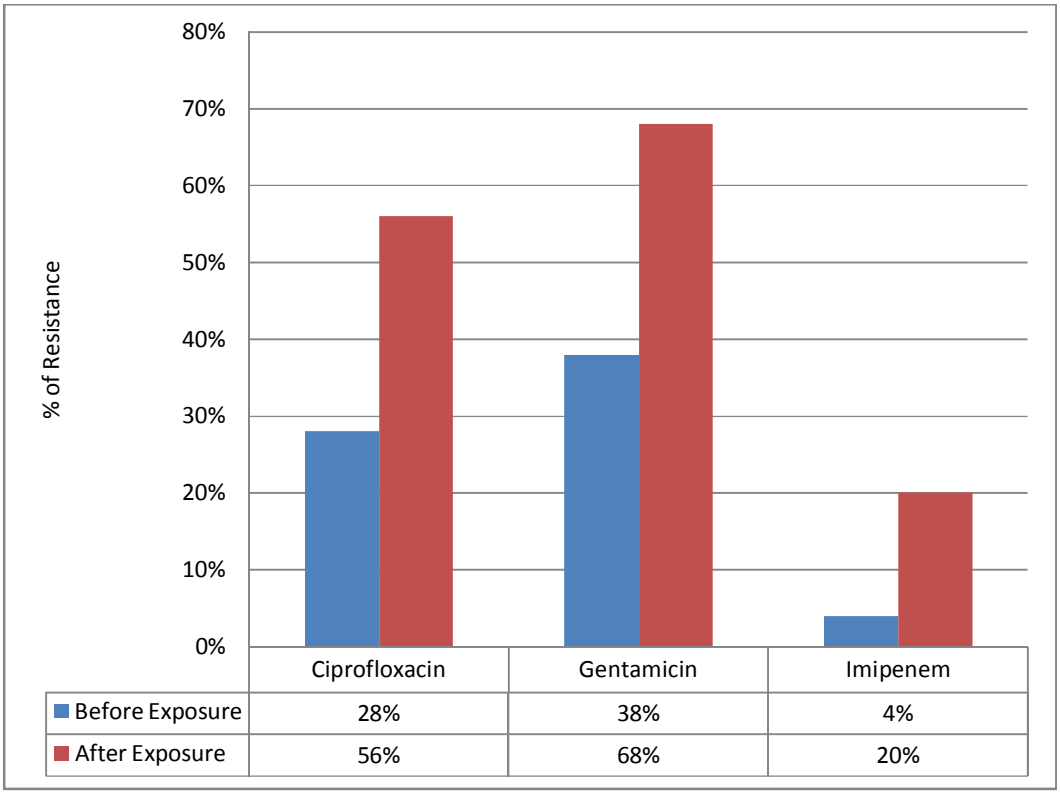
Antimicrobial agents	Before disinfectant exposure	After exposure to disinfectant (Sodium hypochlorite)		After exposure to disinfectant (Benzalkonium chloride)	
	% of resistance	% of resistance	Increase in resistance	% of resistance	Increase in resistance
Ciprofloxacin	20%	42%	22%	50%	30%
Gentamicin	20%	48%	28%	68%	48%
Imipenem	6%	14%	8%	18%	12%

Illustration 1 - Change in Susceptibility pattern (Resistance) of *Pseudomonas aeruginosa* after exposure to Sodium Hypochlorite



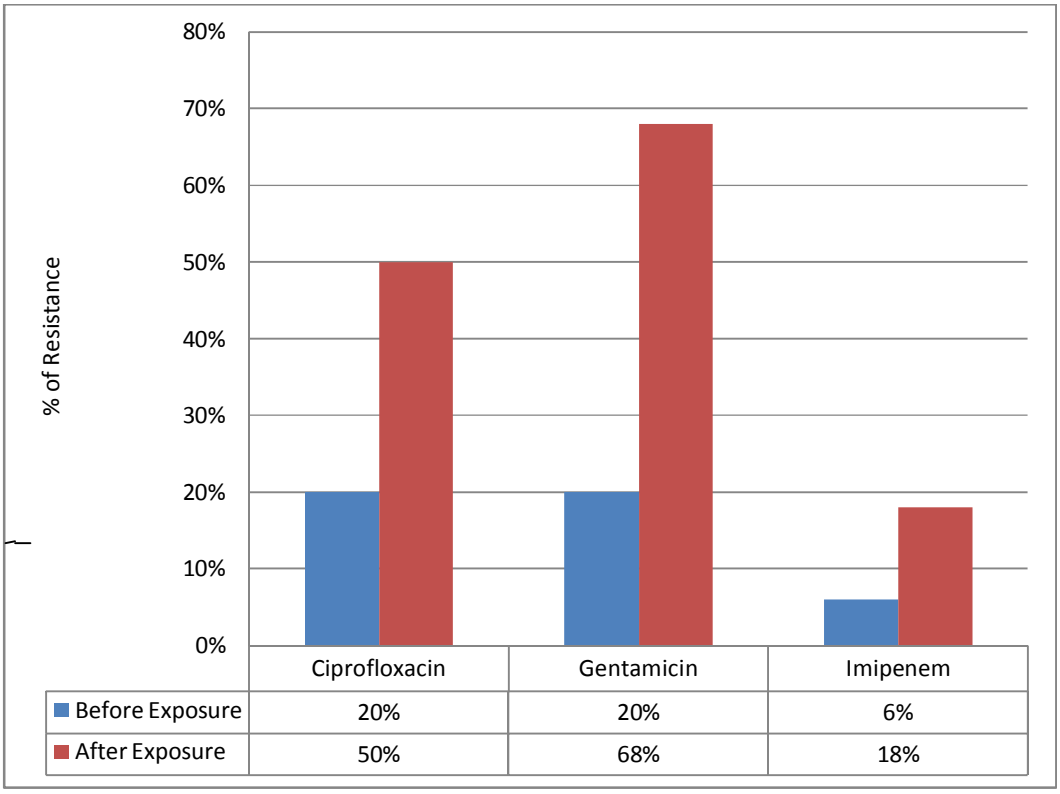
	Ciprofloxacin	Gentamicin	Imipenem
Before Exposure	28%	38%	4%
After Exposure	44%	56%	14%

Illustration 2 - Change in Susceptibility pattern (Resistance) of *Pseudomonas aeruginosa* after exposure to Benzalkonium Chloride



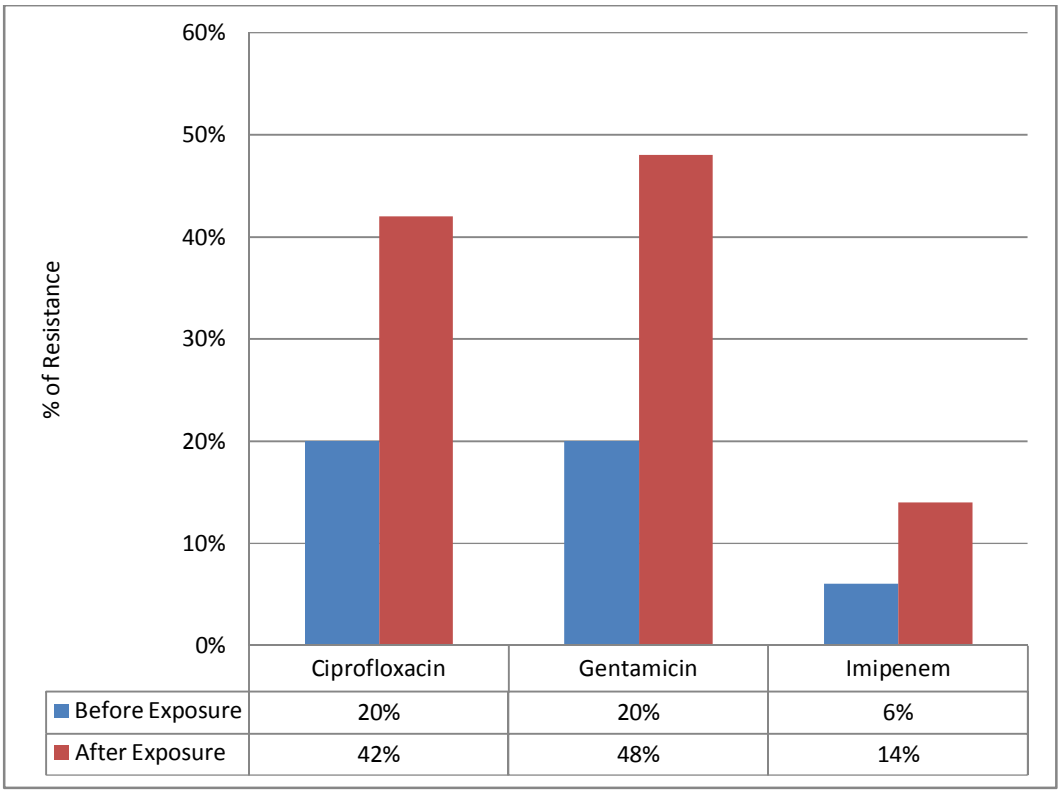
	Ciprofloxacin	Gentamicin	Imipenem
Before Exposure	28%	38%	4%
After Exposure	56%	68%	20%

Illustration 3 - Change in Susceptibility pattern (Resistance) of *Acinetobacter baumannii* after exposure to Benzalkonium Chloride



	Ciprofloxacin	Gentamicin	Imipenem
Before Exposure	20%	20%	6%
After Exposure	50%	68%	18%

Illustration 4 - Change in Susceptibility pattern (Resistance) of *Acinetobacter baumannii* after exposure to Sodium Hypochlorite



After exposure to disinfectants, most of the isolates showed increase in MIC and some isolates showed no change in MIC to the selected antimicrobials. The pattern exhibited by the isolates are shown below (Table 9)

Table 9- Pattern of change in MIC exhibited by the isolates before and after exposure to disinfectants

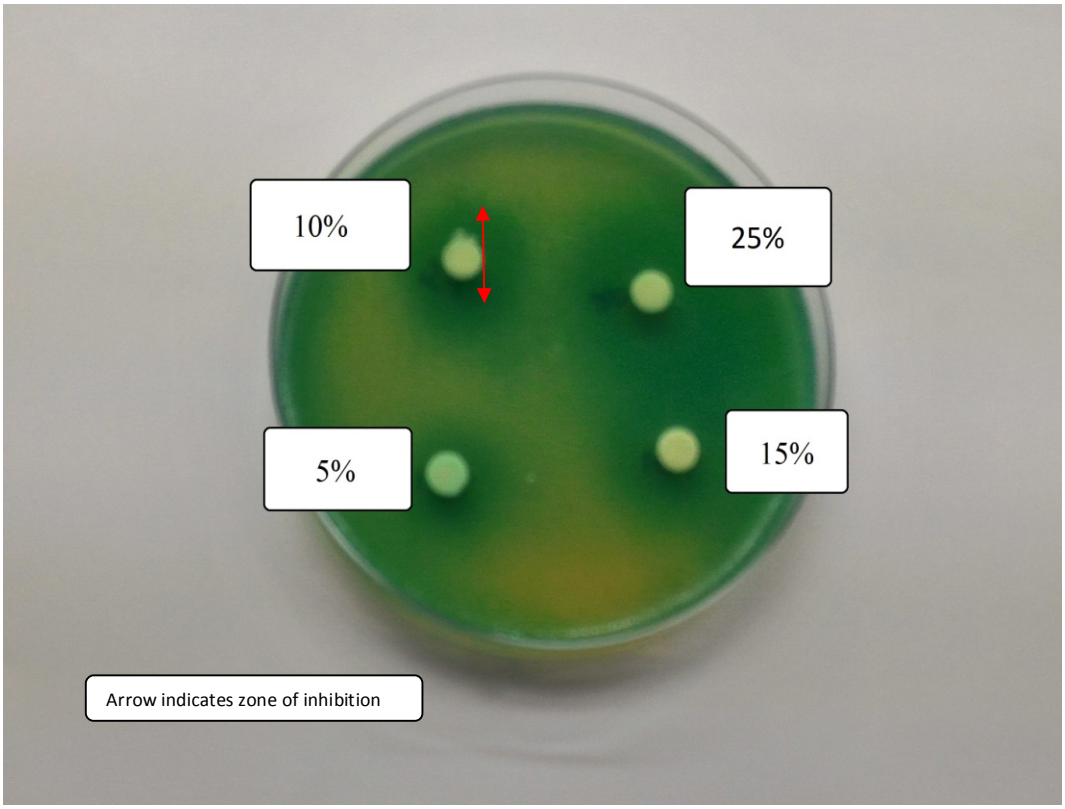
Antimicrobials	*Pseudomonas aeruginosa*				*Acinetobacter baumannii*			
	After exposure to Sodium Hypochlorite		After exposure to Benzalkonium Chloride		After exposure to Sodium Hypochlorite		After exposure to Benzalkonium Chloride	
	No Change in MIC	Increase in MIC	No Change in MIC	Increase in MIC	No Change in MIC	Increase in MIC	No Change in MIC	Increase in MIC
Ciprofloxacin	30%	70%	14%	86%	34%	66%	32%	68%
Gentamicin	50%	50%	28%	72%	22%	78%	40%	60%
Imipenem	20%	80%	34%	66%	24%	76%	22%	78%

EFFECT OF NEEM LEAF EXTRACT ON THE SELECTED ISOLATES

The isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were exposed to varying concentrations of Neem leaf extract (5%, 10%, 15% and 25%). The effect of the extract on the selected bacteria was tested by disc diffusion method on agar plates. All the isolates of *Pseudomonas aeruginosa* were susceptible to 5%, 10%, 15% and 25% whereas the isolates of *Acinetobacter baumannii* did not turn susceptible to the extract.

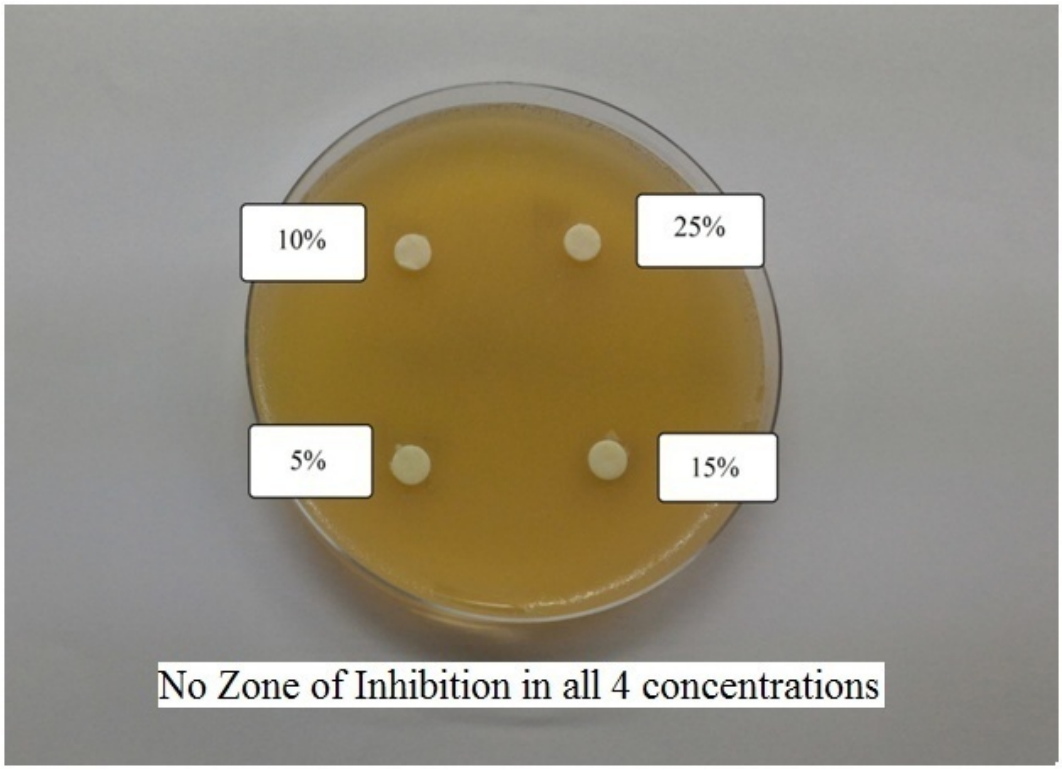
Picture 5-Effect of Neem leaf extract on *Pseudomonas aeruginosa* isolates

(Disc diffusion method)



Picture 6 – Effect of Neem leaf extract on *Acinetobacter baumannii* isolates

(Disc diffusion Method)



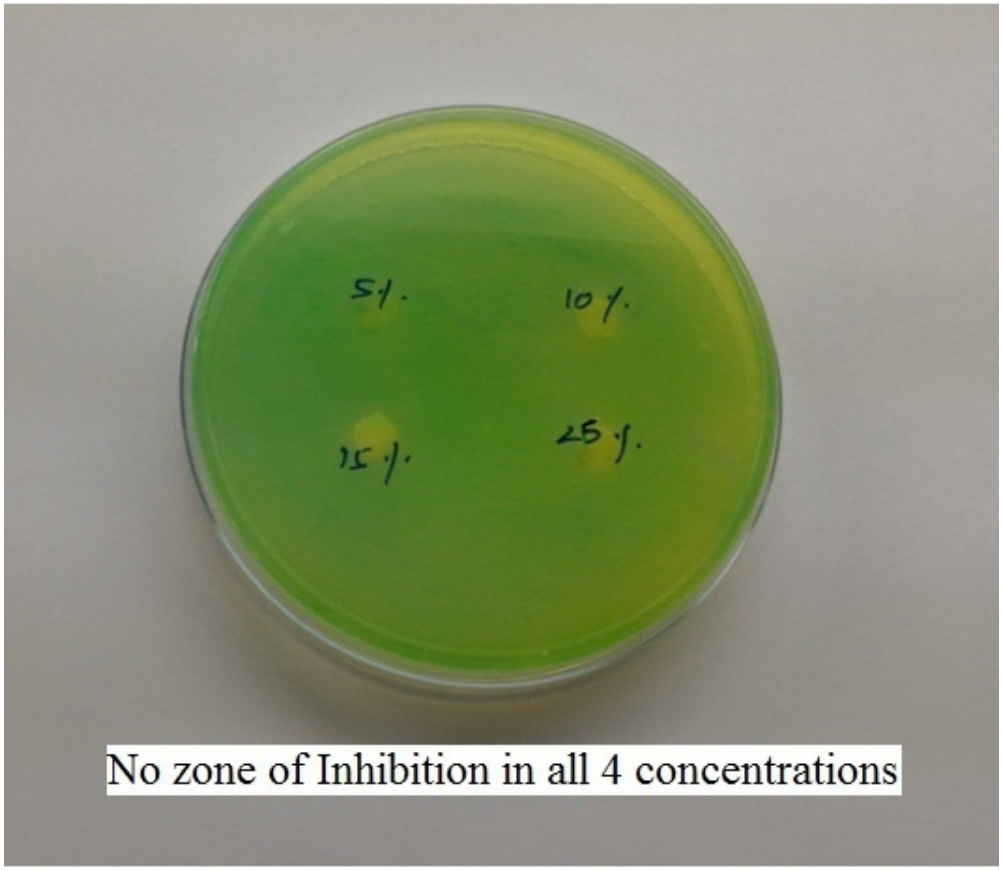
Effect Of Neem leaf extract on *Pseudomonas aeruginosa* after exposure to Disinfectants

After exposure to Sodium hypochlorite and Benzalkonium chloride, all 50 isolates of *Pseudomonas aeruginosa* became resistant to neem leaf extract at 5%, 10%, 15% and 25% concentration to which they were susceptible before (Table 10)

Table 10- Effect of Neem leaf extract on *Pseudomonas aeruginosa* after exposure to Disinfectants

CONCENTRATION OF NEEM LEAF EXTRACT	BEFORE EXPOSURE (ZONE OF INHIBITION)	AFTER EXPOSURE TO SODIUM HYPOCHLORITE (ZONE OF INHIBITION)	AFTER EXPOSURE TO BENZALKONIUM CHLORIDE (ZONE OF INHIBITION)
5%	+	**NIL**	**NIL**
10%	+	**NIL**	**NIL**
15%	+	**NIL**	**NIL**
25%	+	**NIL**	**NIL**

Picture 7- Effect of Neem leaf extract on *Pseudomonas aeruginosa* isolates after disinfectant exposure (Disc diffusion method)



6. Discussion

Infection control is very essential in all hospitals, and in particular, disinfection is a key component of this plan. Many cross infections in the hospital can be prevented by the proper use of disinfectant products and importantly in its correct dilution. This study has explored the effect of bacterial exposure to sub-inhibitory concentrations of disinfectants on the development of antibiotic resistance in these bacteria. In this study, two widely used disinfectants Sodium Hypochlorite and Benzalkonium Chloride and antibiotics like Ciprofloxacin, Gentamicin and Imipenem are tested against clinical isolates, namely, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Ciprofloxacin and Gentamicin are used to treat a number of bacterial infections. Imipenem is an intravenous β lactam antibiotic with a broad spectrum of action.

Sodium Hypochlorite is a widely used chlorine disinfectant. The advantages of Hypochlorites are that they have an extended range of antibacterial action, is inexpensive, fast acting [170] and has a very low incidence of toxicity [171,172,173]. They are used to disinfect the hospital surfaces, spillage of potentially infectious blood and body fluids. Benzalkonium chloride is widely used to disinfect hospital floors, furniture, walls and medical equipments that contact intact skin like blood pressure cuffs. They are also used for disinfecting equipments like endoscopes and intravascular catheters [174,175].

The selected isolates were exposed to varying concentrations of Sodium Hypochlorite ranging from 3% to 0.03% and Benzalkonium Chloride ranging from 4% to 0.06%. But as growth is expected in subinhibitory concentration and

was demonstrated in this study, therefore this concentration was used for exposure studies. In a study done by Majid et al, isolates of *Pseudomonas* were exposed to varying concentrations of Povidone iodine ranging from 5% to 30% and Dichlorometaxylene ranging from 0.1 to 3%, where also, growth in subinhibitory concentration was demonstrated [155].

Initially, the MIC of the antibiotics Ciprofloxacin, Gentamicin and Imipenem were determined to the selected isolates by Agar dilution method in our study as was done in most studies [152,164-168]. In some studies [153,154] Microbroth dilution method was used to calculate the MIC values. Others have used E test to determine the MIC [151,153].

In this study, susceptibility of *Pseudomonas aeruginosa* was 72%, 62% and 92% to Ciprofloxacin, Gentamicin and Imipenem, respectively. This compared well with other studies as regards Ciprofloxacin and Imipenem[138,139], but with Gentamicin it was higher and ranged from 73-94% in some studies[136,137] . However the MICs of Ciprofloxacin, Gentamicin and Imipenem were comparable with other studies in case of *Pseudomonas aeruginosa*[140-143]. All these studies were done during the years from 1997-2003.

Similarly, the susceptibility of *Acinetobacter baumannii* was 80%, 80% and 86% to Ciprofloxacin, Gentamicin and Imipenem, respectively in this study, where as in other studies, the susceptibility to Imipenem was higher (98%) and lower to Ciprofloxacin and Gentamicin [144,149]. As regards the MIC value, it was seen that, they were much higher than our MIC value in case of

Ciprofloxacin[145,146], whereas with Imipenem the MIC values were comparable to that in other studies [147,148]. However, with Gentamicin our MICs were 4 fold higher than that of other studies [147].

Following exposure to disinfectants, MIC for the antibiotics was determined by Agar dilution method. Majority of our study isolates, showed an increase in both the number of isolates becoming resistant as well as an increase in the MICs of antibiotics in already resistant or sensitive strains in many folds. On the other hand, only few isolates showed no change in MIC after exposure.

In our study, 16% of *Pseudomonas* isolates after exposure to Sodium Hypochlorite showed a fourfold rise in MIC to Gentamicin from sensitive to resistance and 8% of the isolates showed a 2 fold increase in MIC for already resistant strains for Ciprofloxacin. Similar results are reported with other bacteria also. In a study done in the year 2011, *Listeria monocytogenes* when exposed to subinhibitory concentrations of Triclosan resulted in a 16 fold increase in its MIC from sensitive to resistance for the antimicrobial Gentamicin[150]. In our study after exposure to Benzalkonium chloride, 12% of *Pseudomonas* isolates showed a sixteen fold rise in MIC for Ciprofloxacin, whereas, a 256 fold rise in MIC to Ciprofloxacin has been reported with Benzalkonium chloride exposure in *Pseudomonas aeruginosa* isolates in other studies[151].

In our study after exposure to Sodium Hypochlorite, 20%, 50% and 20% of *Pseudomonas* isolates showed no change in MIC for the drugs Ciprofloxacin, Gentamicin and Imipenem respectively and among isolates of *Acinetobacter*

baumannii, 32%, 40% and 22% showed no change in MIC for the drugs Ciprofloxacin, Gentamicin and Imipenem respectively after exposure to Benzalkonium chloride. In a study done in 2011, exposure of *Listeria monocytogenes* to subinhibitory concentrations of Hydrogen peroxide and Quaternary ammonium compounds did not alter the antibiotic susceptibility pattern [150].

In our study, after exposure to Sodium Hypochlorite, 4% of *Acinetobacter* isolates showed a twofold rise in MIC to Ciprofloxacin for already resistant strains and after exposure to Benzalkonium chloride, 4% of the isolates showed a 2 fold increase in MIC for already resistant strains for the antimicrobial Ciprofloxacin . In a study done in 2007, strains of *Salmonella* showed 4 fold increase in MIC for Ciprofloxacin after exposure to an aldehyde based disinfectant [152].

In our study none of the isolates showed a decrease in MIC after exposure to disinfectants, whereas in a study, after exposure to Benzalkonium chloride, MIC to drug Ciprofloxacin decreased by 8 folds and also in another study, MIC for isolates of *Burkholderia cepacia* decreased by 2 folds and isolates of *Serratia marcescens* also decreased by 2 folds after exposure to Triclosan [151,154]

This study thus shows that after exposure to subinhibitory concentrations of disinfectants, MIC of resistant strains increased, as well as the strains which were sensitive to the antimicrobials before disinfectant exposure became resistant. The MIC with sensitive isolates also showed an increase in varying fold amongst

the two bacteria tested. As mentioned above, this is consistent with many other studies also[150-157]. This ascertains the fact that lower and thus incorrect dilutions of disinfectants could result in the emergence of multidrug resistant bacteria. In addition, Olukemi and Funmilayo (2011), have also reported an increase in virulence properties of the bacteria studied [156].

In another study, it was proposed that bacteria get adapted to disinfectants which can result in the development of antibiotic resistance. This theory says, that for resistance to be developed in an organism after disinfectant exposure, factors like nature of the antibiotic, biocide and the involved organism are crucial. Many researches are going on in this theory and results of such studies would enhance our knowledge of how the bacteria adapt to disinfectants and develop resistance [157].

In another study conducted by Poole, it was proposed that disinfectant resistant bacteria resulted from structural change in the cell due to disinfectant accumulation, mutations and efflux pump [158] . Another study proposed that repeated use of same disinfectant can result in the emergence of resistant organisms [159].

In our study, isolates of *Pseudomonas aeruginosa* were inhibited by varying concentrations 5%, 10%, 15%, and 25% of neem extract which is similar to other studies [160,161,162]. After exposure to disinfectants, the isolates became resistant to all the above concentrations. The isolates of *Acinetobacter baumannii*

were not susceptible to neem leaf extract in our study which correlates with a study done in the year 2015[163].

There are studies proposing that bacteria possess mechanisms which result in resistance to biocides and antibiotics and also integration of genetic elements in bacteria result in resistance . Such genetic elements carry genes which confer resistance to antibiotics and biocides [169].

Therefore, from all the above studies, it is apparent that, using inappropriate concentrations of disinfectants result in the development of bacteria resistant to antibiotics and biocides. Thus it is evident that disinfectants can train bacteria to resist antimicrobials when disinfectants are used in below optimum concentrations.

7. Summary

The present study conducted to assess the consequence of disinfectant exposure on antibiotic susceptibility pattern of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* and also to assess the susceptibility pattern of the above bacteria to herbal extract (Neem) was carried out in the Department of Microbiology, PSG IMS&R, during the period January 2015 to July 2016.

The above mentioned bacteria (50 isolates each) were collected from various clinical samples received in our Microbiology laboratory. They were exposed to varying concentrations of Sodium Hypochlorite and Benzalkonium Chloride following which their susceptibilities to antibiotics Ciprofloxacin, Gentamicin and Imipenem were studied and compared with the susceptibility pattern exhibited by the isolates before exposure. Testing was done by Agar dilution method.

Neem extract was prepared in varying concentrations and tested for their effect on the selected clinical isolates before and after exposure to disinfectants.

After exposure to subinhibitory concentrations of Sodium Hypochlorite, 70%, 50% and 80% of the isolates of *Pseudomonas aeruginosa* showed increase in MIC folds for the antimicrobial Ciprofloxacin, Gentamicin and Imipenem respectively and 66%, 78% and 76% of the isolates of *Acinetobacter baumannii* showed increase in MIC folds for the antimicrobials Ciprofloxacin, Gentamicin and Imipenem respectively.

After exposure to subinhibitory concentrations of Benzalkonium Chloride, 86%, 72% and 66% of the isolates of *Pseudomonas aeruginosa* showed increase in MIC folds for the antimicrobials Ciprofloxacin, Gentamicin and Imipenem respectively and 68%, 60% and 78% of the isolates of *Acinetobacter baumannii* showed increase in MIC folds for the antimicrobials Ciprofloxacin, Gentamicin and Imipenem respectively.

Paired sample T test was used to calculate the P value for MICs of antibiotics to the selected clinical isolates before and after exposure to disinfectants. For the isolates of *Pseudomonas aeruginosa*, the P values for MICs of the antibiotics Ciprofloxacin, Gentamicin and Imipenem before and after exposure to Sodium Hypochlorite were 0.002, 0.004 and 0.00 respectively which means they are significant. For the same isolates, P value was 0.00 for MICs of all the above antibiotics before and after exposure to Benzalkonium Chloride which means they are significant.

Similarly for the isolates of *Acinetobacter baumannii*, P value for all the above said antibiotics before and after treatment with Sodium Hypochlorite was 0.00. For the same isolates, P value for MICs of all the selected antibiotics before and after treatment with Benzalkonium Chloride was 0.00. This shows that the increase in MIC values before and after treatment with both the disinfectants are significant.

The clinical isolates when exposed to varying concentrations of Neem leaf extract 5%, 10%, 15% and 25% , only isolates of *Pseudomonas aeruginosa* were susceptible to all the above concentrations and none of the isolates of *Acinetobacter baumannii* were susceptible. When the isolates of Pseudomonas were tested for susceptibility to the extract after exposure to the chosen disinfectants, they turned resistant to all the above concentrations to which they were sensitive before.

Therefore it is evident from this study that usage of disinfectants in incorrect dilutions can result in the emergence of bacteria resistant to antibiotics and biocides which is a global crisis and needs to be addressed.

8. Conclusion

- The conclusion we have drawn from this study is that, disinfectants when used in inappropriate dilutions result in the development of multidrug resistant bacteria.

- Two common multidrug resistant bacteria namely *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were selected in this study. Both the isolates showed change in antibiotic susceptibility pattern (Increase in resistance) after exposure to disinfectants Benzalkonium Chloride and Sodium Hypochlorite. The susceptibility pattern was altered for all three antibiotics chosen namely Ciprofloxacin, Gentamicin and Imipenem. Many of our findings have correlated well with other studies.

- Isolates of *Pseudomonas aeruginosa* were susceptible to the prepared Neem leaf extract and isolates of *Acinetobacter baumannii* were not susceptible to the extract. After exposure to the disinfectants, *Pseudomonas* isolates turned resistant to the herbal extract to which they were susceptible before.

- The change in susceptibility pattern for all the three antibiotics before and after exposure to disinfectants were significant (p value < than 0.05)

- Thus this study shows the association of disinfectant usage in incorrect dilutions with the development of resistance to antibiotics and biocides. This emphasizes the need for timely action to be taken to prevent the emergence of an alarming increase in resistance among organisms to biocides and antibiotics.

- Neem tree yields many products which are medically significant and when properly used, they can result in the invention of newer drugs which are cheap and safe.

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ABBREVIATIONS

MIC - Minimal Inhibitory Concentration

WHO - World Health Organisation

EFSA - European Food Safety Authority

LPS - Lipopolysaccharide

MDR - Multidrug Resistant

HAI - Hospital Acquired Infection

CDC - Centers for Disease Control and prevention

OSHA - Occupational Safety and Health Administration

EPA - Environmental Protection Agency

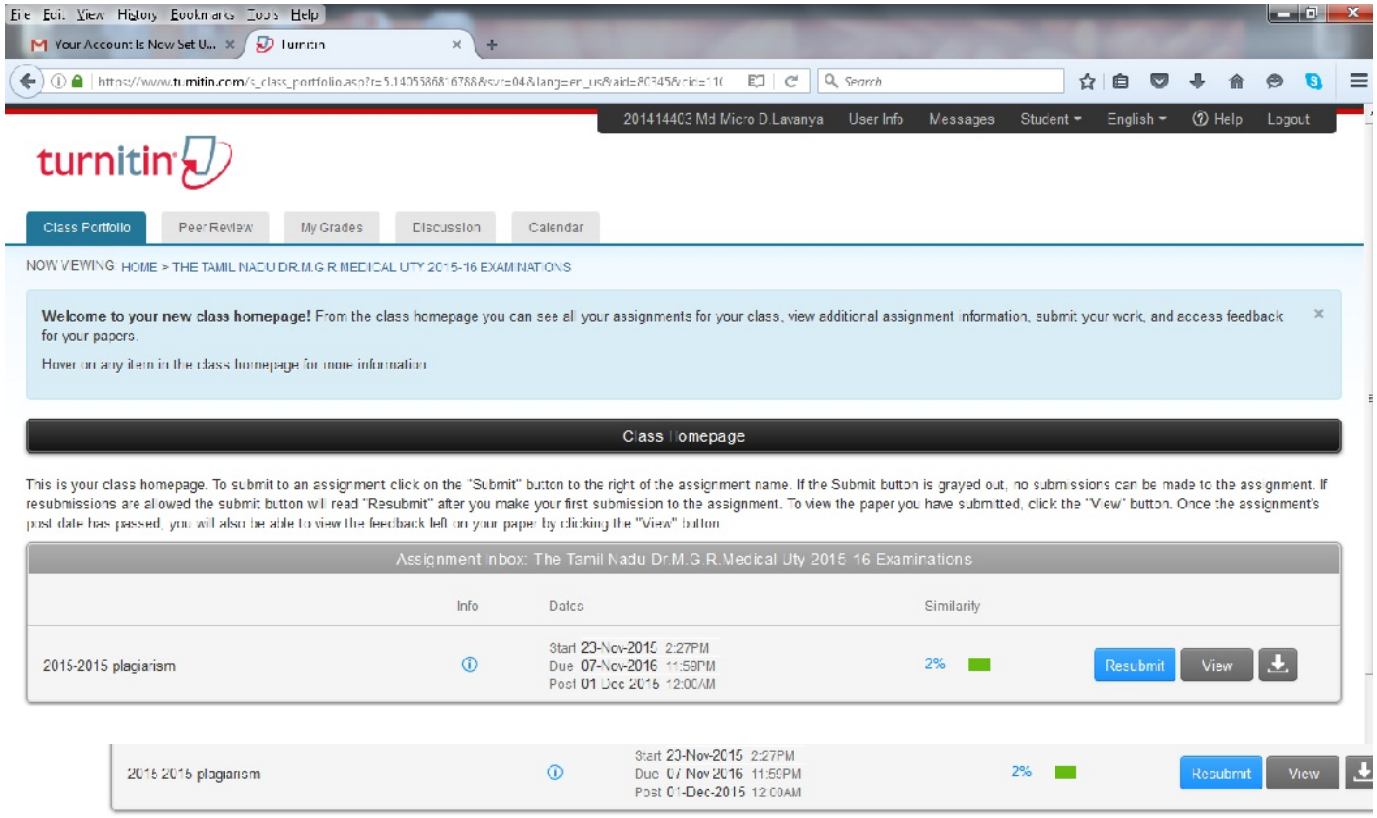
ICU - Intensive Care Unit

UTI - Urinary Tract Infection

ENT - Ear, Nose and Throat

CFU - Colony Forming Units

Annexures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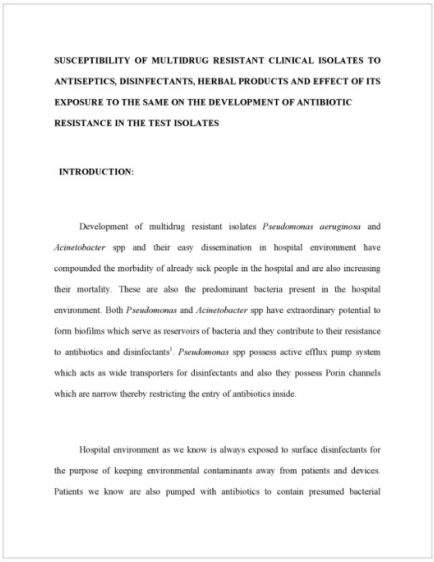

SUSCEPTIBILITY OF MULTIDRUG RESISTANT CLINICAL ISOLATES TO ANTISEPTICS, DISINFECTANTS, HERBAL PRODUCTS AND EFFECT OF ITS EXPOSURE TO THE SAME ON THE DEVELOPMENT OF ANTIBIOTIC RESISTANCE IN THE TEST ISOLATES

INTRODUCTION:

Development of multidrug resistant isolates *Pseudomonas aeruginosa* and *Acinetobacter* spp and their easy dissemination in hospital environment have compounded the morbidity of already sick people in the hospital and are also increasing their mortality. These are also the predominant bacteria present in the hospital environment. Both *Pseudomonas* and *Acinetobacter* spp have extraordinary potential to form biofilms which serve as reservoirs of bacteria and they contribute to their resistance to antibiotics and disinfectants[1]. *Pseudomonas* spp possess active efflux pump system which acts as wide transporters for disinfectants and also they possess Porin channels which are narrow thereby restricting the entry of antibiotics inside.

Hospital environment as we know is always exposed to surface disinfectants for the purpose of keeping environmental contaminants away from patients and devices. Patients we know are also pumped with antibiotics to contain presumed bacterial

status report as decided by the IHEC.

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

To
Dr D Lavanya
Postgraduate
Department of Microbiology
PSG IMS & R
Coimbatore

Ref: Project No. 14/393

Date: December 12, 2014

Dear Dr Lavanya,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 02.12.2014 to conduct the research study entitled "*Susceptibility of multidrug resistant clinical isolates to antiseptics, disinfectants, herbal products and effect of its exposure to the same on the development of antibiotic resistance in the test isolates*" during the IHEC meeting held on 05.12.2014.

The following documents were reviewed and approved:

1. Project Submission form
2. Study protocol
3. Confidentiality statement
4. Application for waiver of consent
5. Current CVs of Principal investigator, Co-investigators
6. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the meeting held on 05.12.2014 at IHEC Secretariat, PSG IMS & R between 10.00 am and 11.00 am:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Dr. P. Sathyan (Chairperson, IHEC)	DO, DNB	Clinician (Ophthalmology)	Male	No	Yes
2	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
3	Dr. S.Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
4	Dr. D. Vijaya	M Sc, Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

The study is approved in its presented form. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the ICH-GCP/ICMR/Schedule Y guidelines. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of status report as decided by the IHEC.

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee
Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

Following points must be noted:

1. IHEC should be informed of the date of initiation of the study
2. Status report of the study should be submitted to the IHEC every 12 months
3. PI and other investigators should co-operate fully with IHEC, who will monitor the trial from time to time
4. At the time of PI's retirement/intention to leave the institute, study responsibility should be transferred to a colleague after obtaining clearance from HOD, Status report, including accounts details should be submitted to IHEC and extramural sponsors
5. In case of any new information or any SAE, which could affect any study, must be informed to IHEC and sponsors. The PI should report SAEs occurred for IHEC approved studies within 7 days of the occurrence of the SAE. If the SAE is 'Death', the IHEC Secretariat will receive the SAE reporting form within 24 hours of the occurrence
6. In the event of any protocol amendments, IHEC must be informed and the amendments should be highlighted in clear terms as follows:
   a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b. Alteration in the budgetary status should be clearly indicated and the revised budget form should be submitted
   c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval
   d. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented
   e. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IHEC and only then can they be implemented
   f. Any deviation-Violation/waiver in the protocol must be informed to the IHEC within the stipulated period for review
7. Final report along with summary of findings and presentations/publications if any on closure of the study should be submitted to IHEC

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Thanking You,

Yours Sincerely,

12.12.14

Dr S Bhuvaneshwari
Member-Secretary
Institutional Human Ethics Committee

SECRETARY
PSG IMS&R
COIMBATORE-641004.
INSTITUTIONAL HUMAN ETHICS COMMITTEE

भारत सरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
MINISTRY OF ENVIRONMENT, FORESTS & CLIMATE CHANGE
भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA
दक्षिणी क्षेत्रीय केन्द्र / Southern Regional Centre
टी.एन.ए.यू कैम्पस / T.N.A.U. Campus
लाउली रोड / Lawley Road
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टेलीफक्स/ Telefax: 0422- 2432835
ई-मैल /E-mail id: sc@bsi.gov.in
bsisc@rediffmail.com

सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2016/Tech. /570 दिनांक/Date: 12th April 2016

सेवा में / To

Dr. D. Lavanya
II Year MD Post Graduate
Department of Microbiology
PSG IMS&R
Coimbatore – 641 004

महोदया/Madam,

The plant specimen brought by you for identification is identified as *Azadirachta indica* A. Juss. - MELIACEAE. The identified specimen is returned herewith for preservation in their college/ Department/ Institution Herbarium.

धन्यवाद/Thanking you,

भवदीय/Yours faithfully,

12/4/16

(डॉ. जी.वी.एस. मूर्ति /Dr. G.V.S. Murthy)
वैज्ञानिक 'जी' एवं कार्यालय अध्यक्ष /
Scientist 'G' & Head of Office

वैज्ञानिक 'जी' एवंकार्यालयअध्यक्ष
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Southern Regional Centre
कोयम्बत्तूर / Coimbatore - 641 003.